UNIVERSIDADE FEDERAL DO PARANÁ

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SOLUÇÕES PARA BIORREFINARIAS DE POLPA CÍTRICA

CURITIBA

2015

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Tese apresentada como requisito parcial à obtenção de título de Doutor em Ciências (Bioquímica), no Curso de Pós-Graduação em Bioquímica da Universidade Federal do Paraná.

Orientador: Prof. Dr. David Alexander Mitchell
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CURITIBA

2015

TERMO DE APROVAÇÃO

ALESSANDRA BIZ

Soluções para Biorrefinarias de Polpa Cítrica

Tese aprovada como requisito parcial para obtenção do grau de Doutor no curso de Pós-Graduação em Ciências-Bioquímica, Setor de Ciências Biológicas, Universidade Federal do Paraná, pela seguinte banca examinadora:

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Curitiba, 18 de novembro de 2015.

RESUMO

Biorrefinaria é um novo conceito que abrange as instalações industriais que utilizam a biomassa como matéria-prima para a produção de intermediários químicos, combustíveis e energia, analogamente às refinarias de petróleo. Uma matéria prima que apresenta oportunidades para ser utilizada no Brasil é a polpa cítrica, que é o resíduo do processamento do suco de laranja. Este resíduo é produzido em grandes quantidades no Brasil, que é o maior produtor mundial de sucos. Atualmente, a polpa cítrica não tem valor comercial importante, já que os custos de secagem comprometem as margens de revenda como ração animal, que é a destinação típica para este resíduo. Por outro lado, a polpa cítrica é uma fonte de limoneno e pectina, que já têm processos de extração estabelecidos, e os carboidratos da polpa podem ser transformadas em etanol e alguns ácidos com alto valor agregado, como o ácido D-galacturônico, o ácido L-galactônico, o ácido múcico e o ácido ascórbico. O objetivo geral desta tese de doutorado foi propor soluções para três problemas das biorrefinarias de polpa cítrica, a falta de conhecimento sobre a hidrólise enzimática da pectina, o custo alto das pectinases que são usadas na hidrólise enzimática da pectina e a incapacidade de *Saccharomyces cerevisiae* produzir etanol a partir do ácido galacturônico liberado. Na primeira parte do trabalho, a hidrólise enzimática da pectina foi caracterizada, com intuito de entender a lentidão na liberação dos açúcares. Ficou demonstrado, pela primeira vez, que existe uma desaceleração significativa na liberação de açúcares redutores já nos primeiros minutos de hidrólise de pectina por complexos pectinolíticos. Foi mostrado que, com esta falta de linearidade no início da reação, o tempo de incubação escolhido para um ensaio tem grande efeito na atividade medida. De fato, é destacada a necessidade de estabelecer um protocolo padrão para a determinação da atividade pectinolítica, em termos da temperatura, da concentração de pectina e do tempo de incubação. Somente com esta padronização vai ser possível comparar processos de produção de pectinases realizados por grupos diferentes. Na segunda parte do trabalho, foi desenvolvido um processo, em escala piloto, para a produção de pectinases em fermentação em estado sólido, com intuito de baixar o custo destas enzimas. O processo foi realizado com 15 Kg (base seca) de um substrato composto por polpa cítrica e bagaço de cana, na razão de 51,6 a 48,4, (m/m). O uso de uma proporção alta de bagaço propiciou um leito de alta porosidade e, desta maneira, o problema de superaquecimento do leito, que é comum em processos de fermentação em estado sólido, foi completamente evitado. Também foi demonstrado que, no final da fermentação, o sólido pode ser secado e adicionado diretamente a uma solução de pectina para efetuar sua hidrólise; desta maneira, os custos de recuperação e concentração das pectinases são evitados. Na terceira parte do trabalho, foi construída uma linhagem de *S. cerevisiae* capaz de consumir o ácido D-galacturônico. Isto foi conseguido pela integração de uma via heteróloga, proveniente de fungos filamentosos, de catabolismo de ácido D-galacturônico. Este estudo representa o primeiro passo na construção de uma cepa de *S. cerevisiae* capaz de produzir etanol a partir de ácido D-galacturônico, sendo que este açúcar chega a compor cerca de 18% do total de açúcares em hidrolisados de polpa cítrica.

Palavras-chave: Biorrefinarias, polpa cítrica, ácido D-galacturônico, etanol, pectinases, escalonamento, fermentação no estado sólido.

ABSTRACT

Biorefineries are industrial installations that utilize biomass as a raw material for the production of chemicals, fuels and energy, in a manner analogous to petroleum refineries. One raw material that has the potential to be utilized in Brazil is citrus pulp, the residue of the processing of orange juice. This residue is produced in large quantities in Brazil, which is the world's largest producer of orange juice. Currently, citrus pulp has no significant commercial value; it is typically used as a supplement for cattle feed, but the drying costs mean that the return is minimal. However, citrus pulp is a source of limonene and pectin, for which extraction processes are already established. Further, the carbohydrates in the pulp can be processed into ethanol and some acids with high added value, such as D-galacturonic acid, L-galactonic acid, mucic acid and ascorbic acid. The overall objective of this thesis was to propose solutions to three problems faced by citrus pulp biorefineries: the lack of knowledge about enzymatic hydrolysis, the high cost of the pectinases that are used in the enzymatic hydrolysis of pectin and the inability of *Saccharomyces cerevisiae* to produce ethanol from the galacturonic acid released in such hydrolysis processes. In the first part of the work, the enzymatic hydrolysis of pectin was characterized, with the aim of understanding why the release of sugars is so slow. For the first time, it was demonstrated that there is a significant deceleration in the release of reducing sugars within the first minutes of pectin hydrolysis by pectinases. It was shown that, with this lack of linearity at the start of the reaction, the incubation time that is chosen for an assay of pectinase activity has a large effect on the activity that is measured. In fact, the thesis raises the need to establish a standard protocol for the determination of pectinolytic activity, in terms of temperature, pectin concentration and time of incubation. Only with such standardization will it be possible to compare pectinase production processes proposed by different research groups. In the second part of the work, a pilot-scale process was developed for the production of pectinases in solid-state fermentation, with the aim of lowering the cost of these enzymes. The process was conducted with 15 kg (dry basis) of a substrate composed of citrus pulp and sugarcane bagasse, in a ratio of 51.6 to 48.4 (by mass). The use of a high proportion of bagasse ensured the bed had a high porosity and, consequently, the problem of overheating of the bed, which is common in solid-state fermentation processes, was completely avoided. It was also shown that, at the end of the fermentation, the solid can be dried and added directly to a pectin solution to effect its hydrolysis; thereby avoiding the need to recover and concentrate the pectinases. In the third part of the work, a strain of *S. cerevisiae* was constructed that is capable of consuming D-galacturonic acid. This was achieved by integration of a heterologous pathway for the catabolism of D-galacturonic acid from filamentous fungi. This study represents the first step in the construction of a strain of *S. cerevisiae* capable of producing ethanol from D-galacturonic acid, which represents about 18% of the total sugars in citrus pulp hydrolysates.

Keywords: Biorefineries, citrus pulp, D-galacturonic acid, ethanol, pectinases, scale-up, solid-state fermentation.

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LISTA DE ABREVIATURAS

AKA – Academia da Finlândia

CEPAGRO – Centro de Estudos e Promoção da Agricultura de Grupo

CitrusBR – Associação Nacional de Exportadores de Sucos Cítricos

CNPq – Conselho Nacional de Desenvolvimento Científico e Tecnológico

FCOJ – Suco de laranja concentrado congelado

FDCA – Ácido 2,5 furandicarboxílico

FES – Fermentação no estado sólido

FS – Fermentação submersa

HG – Homogalacturonana

IBGE – Instituto de Geografia e Estatística

NREL – Laboratório Nacional de Energia Renovável

PEL – Pectina liase

PGA – Poligalacturonase

PLY – Pectato liase

PME – Pectina metil esterase

RG – Ramnogalacturonana

VTT – Centro de Pesquisas Técnicas da Finlândia

XPG – Exo-poligalacturonase

SUMÁRIO

Capítulo 1

1. Introdução

Contexto da tese de doutorado no âmbito do projeto de cooperação AKA/CNPq

Esta tese de doutorado é parte integrante de um projeto de cooperação em andamento entre o Laboratório de Tecnologia Fermentativa e Enzimática do Departamento de Bioquímica e Biologia Molecular da UFPR e o grupo de Biologia Sintética da VTT (Centro de Pesquisas Técnicas da Finlândia). O projeto de colaboração foi aprovado pelo CNPq Edital n.º 68/2008 (Convênio Bilateral de Cooperação Internacional com a Academia da Finlândia - AKA) e renovado no Edital N° 30/2012. O objetivo principal desta parceria é desenvolver uma tecnologia adequada à conversão de resíduos ricos em pectina, como a polpa cítrica, a ácido D-galacturônico, etanol e intermediários de síntese química, para a implantação em biorrefinarias.

Histórico dos trabalhos feitos no âmbito do projeto de cooperação

A VTT é uma instituição governamental de pesquisa multi-tecnológica, com foco no desenvolvimento de produtos e processos inovadores, com potencial para o depósito de patentes, além da publicação de artigos em ciência básica e aplicada. O grupo de pesquisa Biologia Sintética da VTT faz parte do departamento “Cell Factory”, e conta com uma equipe de especialistas na área de biologia molecular e engenharia metabólica. Esse grupo começou o trabalho na área com a elucidação da via completa do catabolismo de ácido D-galacturônico em *Aspergillus niger* e *Trichoderma reseei* (HILDITCH *et al*., 2007). O grupo também contribuiu para a elucidação da via de catabolismo da galactose em *A. niger* (MOJZITA *et al*., 2012) e construíram uma levedura transgênica capaz de utilizar a arabinose para a produção de etanol (RICHARD *et al*., 2003). Já o grupo do Laboratório de Tecnologia Fermentativa e

Enzimática da UFPR conta com amplo conhecimento em processos fermentativos em estado sólido, modelagem matemática de crescimento de fungos e de cinética enzimática, produção enzimática de biodiesel e hidrólise de substratos pécticos. Portanto, os conhecimentos técnicos dos dois grupos se complementam para o desenvolvimento de processos de biorrefinarias de polpa cítrica.

Essa parceria entre os grupos de pesquisa está sendo muito proveitosa. Ao todo, foram três visitas do grupo finlandês ao laboratório do Brasil. Essas visitas foram voltadas, principalmente, à discussão dos projetos em comum, ao treinamento dos pesquisadores finlandeses nas técnicas de fermentação em estado sólido (FES), além da introdução dos pesquisadores brasileiros às técnicas de engenharia metabólica, através de um curso ministrado pelos pesquisadores finlandeses. Além disso, a autora desta tese e mais uma aluna de doutorado do grupo fizeram o estágio sanduíche na VTT, em Espoo - Finlândia. Uma aluna de iniciação científica também fez estágio na VTT, pelo programa Ciências Sem Fronteiras.

Esse projeto de cooperação também resultou na publicação de cinco artigos em periódicos internacionais, listado a seguir:

1. Pitol L. O., **Biz A**., Mallmann E., Krieger N., Mitchell D. A. Production of pectinases by solid-state fermentation in a pilot-scale packed-bed bioreactor. Chemical Engineering Journal 283:1009. 2016.
2. Kuivanen J., Pentilä M., Richard P. Metabolic engineering of the fungal D-galacturonate pathway for L-ascorbic acid production. Microbial Cell Factories. 14(1):2. 2015. doi: 10.1186/s12934-014-0184-2.
3. **Biz A.**, Farias, F. C., Motter F. A., De Paula D. H., Richard P., Krieger N., Mitchell D. A. Pectinase Activity Determination: An Early Deceleration in the Release of Reducing Sugars Throws a Spanner in the Works! PLoS One. 9. 2014. Article e109529.
4. Kuivanen J., Pentilä M., Richard P. The *yjjN* of *E. coli* codes for an L-galactonate dehydrogenase and can be used for quantification of L-galactone and L-gulonate. Applied Biochemistry and Biotechnology. 173 (7) 1829-1835. 2014. doi: 10.1007/s12010-014-0969-0

5. Kuivanen J., Dantas H., Mojzita M., Mallmann E., **Biz A.**, Krieger N., Mitchell D. A. Richard P. Conversion of orange peel to L-galactonic acid in a consolidated process using engineered strains of *Aspergillus niger*. AMB Express. Article 33 (4). 2014.

Esta tese está dividida em quatro capítulos. O primeiro capítulo dá base para o entendimento dos principais desafios relacionados à viabilização de biorrefinarias de polpa cítrica. O segundo capítulo é o manuscrito do primeiro artigo publicado no âmbito desta tese, que é uma análise da hidrólise enzimática da pectina em curtos intervalos de tempo, que levanta questionamentos para o método de determinação da atividade pectinolítica. O terceiro capítulo é o manuscrito de um artigo em fase de submissão, que trata da produção de pectinases por fermentação no estado sólido em escala piloto. O quarto capítulo também é o manuscrito de um artigo em fase de submissão, e trata do desenvolvimento de uma levedura geneticamente modificada capaz de utilizar o ácido D-galacturônico, um açúcar muito abundante em resíduos pécticos, para a produção de etanol. Portanto, o conjunto destes quatro capítulos é um pequeno incremento no entendimento dos principais desafios de biorrefinarias de polpa cítrica, e visa contribuir para o desenvolvimento de soluções que permitam viabilizar essa indústria.

2. Revisão Bibliográfica

2.1 Biorrefinarias de polpa cítrica no cenário brasileiro

Biorrefinaria é um conceito que surgiu nos últimos 15 anos e se refere às instalações industriais, análogas às refinarias de petróleo, que visam à conversão de biomassa de diferentes fontes em diversos produtos, tais como combustíveis, energia e *commodities* industriais, além de intermediários químicos (OHARA, 2003). O Brasil tem um enorme potencial para a instalação de biorrefinarias, com o desdobramento

da cadeia produtiva e tecnológica do etanol, e por possuir uma grande vocação agroindustrial, com uma das maiores áreas de terras cultivadas do mundo, 71 milhões de hectares, e com uma das maiores produções agrícolas mundiais, 200 milhões de toneladas (estimada para cereais, leguminosas e oleaginosas em 2015, IBGE/CEPAGRO 2015).

Um dos cultivares que têm grande importância para a economia agrícola do Brasil é a laranja. Na safra 2014/2015, estima-se que foram produzidas 12,6 milhões de toneladas de laranja, dos quais 84% se destinaram à produção de FCOJ (suco de laranja concentrado a 65°BRIX e congelado) (estimativa, CitrusBR 2015). Esta produção coloca o Brasil na posição de maior produtor e exportador mundial de suco de laranja, com 54% do mercado. O segundo maior produtor é os Estados Unidos, com 25%. Na safra de 2013/2014, foram produzidas 1,71 milhões de toneladas de FCOJ por esses dois países (USDA, 2015). No entanto, quase 50% do peso da laranja é resíduo, denominado polpa cítrica. É possível estimar que foram produzidas 2,30 milhões de toneladas de polpa cítrica (peso seco) apenas no Brasil e nos Estados Unidos, admitindo que a massa de polpa cítrica gerada é 1,35 vezes a massa de FCOJ (considerando que o suco é extraído a 12°BRIX e que a polpa cítrica possui 75% de umidade)

A polpa cítrica é tipicamente utilizada na complementação da ração do gado bovino (Fig. 1). Como a polpa cítrica tem pelo menos 75% de umidade, a secagem acaba minimizando a margem de lucro desta atividade. Muitas vezes, os criadores de gado são convidados a retirar gratuitamente o produto úmido, na tentativa de reduzir o gasto com a destinação deste resíduo sólido (MFRURAL, 2015). Além disso, a polpa cítrica não pode ser o único componente para a ração, devido ao baixo conteúdo proteico, e à presença de fatores anti-nutricionais (TEIXEIRA, 1997). No entanto, a polpa cítrica é uma fonte importante de carboidratos, principalmente de pectina, que podem ser convertidos em produtos de maior valor agregado em biorrefinarias.

Figura 1. Polpa cítrica. Fonte: http://renewablespirits.com

Ainda nos anos 90, a crescente quantidade de resíduos gerados pelo processamento do suco de laranja nos Estados Unidos levou à uma expansão do número de estudos com o objetivo de adicionar valor a este resíduo. Estes estudos estavam focados principalmente na hidrólise química e enzimática da polpa cítrica e à produção de etanol a partir dos açúcares do hidrolisado. Estes estudos foram liderados principalmente pelos pesquisadores do NREL (*National Renewable Energy Laboratory* – Laboratório de Energia Renovável Nacional) (GROHMANN e BALDWIN., 1992; GROHMANN, CAMERON e BUSLIG, 1994; WILKINS, WIDMER e GROHMANN, 2007; WIDMER, ZHOU e GROHMANN, 2010). Mais tarde, o crescente aumento no preço do petróleo no começo dos anos 2000 estimulou um aumento nos esforços para o desenvolvimento e implementação de biorrefinarias. Este movimento incluiu algumas propostas para biorrefinarias de polpa cítrica (LOPEZ, LI e THOMPSON, 2010; LOHRASBI *et al.* 2010; RIVAS-CANTU, JONES e MILLS, 2013), que em linhas gerais, propõem o aproveitamento da polpa cítrica para a produção de D-limoneno e pectina como produtos principais, e a produção de etanol e biogás como produtos secundários, visando o aproveitamento total da biomassa.

LOPEZ, LI e THOMPSON (2010), por exemplo, propuseram a extração da pectina e os óleos essenciais a partir dos resíduos de citros, como a principal fonte de faturamento. Os carboidratos restantes deveriam ser, então, hidrolisados e fermentados principalmente à etanol, ou usados para a produção de enzimas ou ainda para a produção de proteínas para enriquecimento de rações (*single cell protein*) (Fig 2). A pectina é usada como agente gelificante e estabilizante na indústria alimentícia,

e tem um valor de venda apreciável, 8 $/lb (BOMGARDNER, 2013). Por outro lado, o potencial de produção de pectina alimentícia a partir da polpa cítrica, mesmo considerando somente a produção no Brasil, superaria de longe a demanda mundial, que é de apenas 53 mil toneladas/ano (BOMGARDNER, 2013). Baseado no potencial de extração de 14,4% (m/m, pectina/polpa cítrica seca) relatado por MAMMA *et al.* (2008), e na massa total de polpa cítrica produzida no Brasil, de 1,6 milhões de toneladas (calculado de USDA, 2015), poderiam ser obtidas 236 mil toneladas de pectina. Portanto, a sugestão de LOPEZ, LI e THOMPSON (2010), de produzir pectina como um produto final, poderia não ser economicamente viável. Desta maneira a hidrólise da pectina contida na polpa cítrica para a produção de combustíveis e materiais também deveria ser um processo central em biorrefinarias de polpa cítrica.

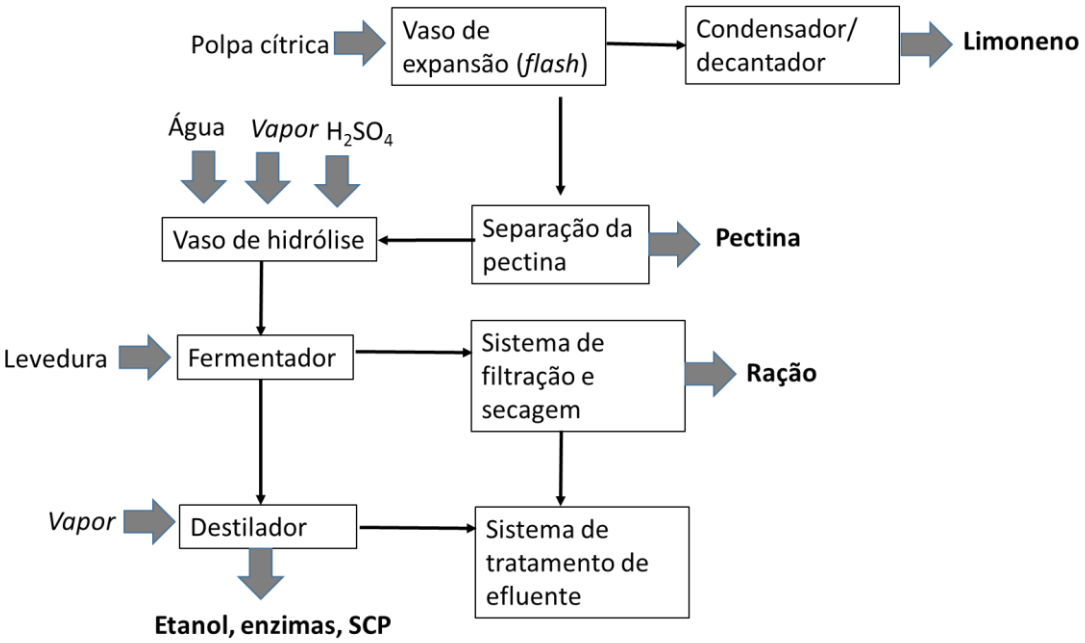

Figura 2. Descrição do processo da biorrefinaria proposta por LOPEZ, LI e THOMPSON (2010).

LOHRASBI *et al.* (2010) propôs uma outra concepção de biorrefinaria de polpa cítrica (Fig. 3) e fez a análise econômica do processo proposto. Em vez de ter a extração da pectina como processo principal, como proposto por LOPEZ, LI e THOMPSON (2010), eles propuseram a hidrólise da polpa cítrica para a produção de etanol como atividade central da planta. Neste processo, o limoneno seria primeiramente extraído, já que possui um alto valor agregado, e, ainda, atrapalha o

processo fermentativo. Em seguida, a polpa cítrica passaria por processo de hidrólise, e posteriormente, o hidrolisado seria fermentado com *Saccharomyces cerevisiae*. O etanol seria separado, e a vinhaça resultante seria utilizada para produção de biogás, que alimentariam as caldeiras da biorrefineria.

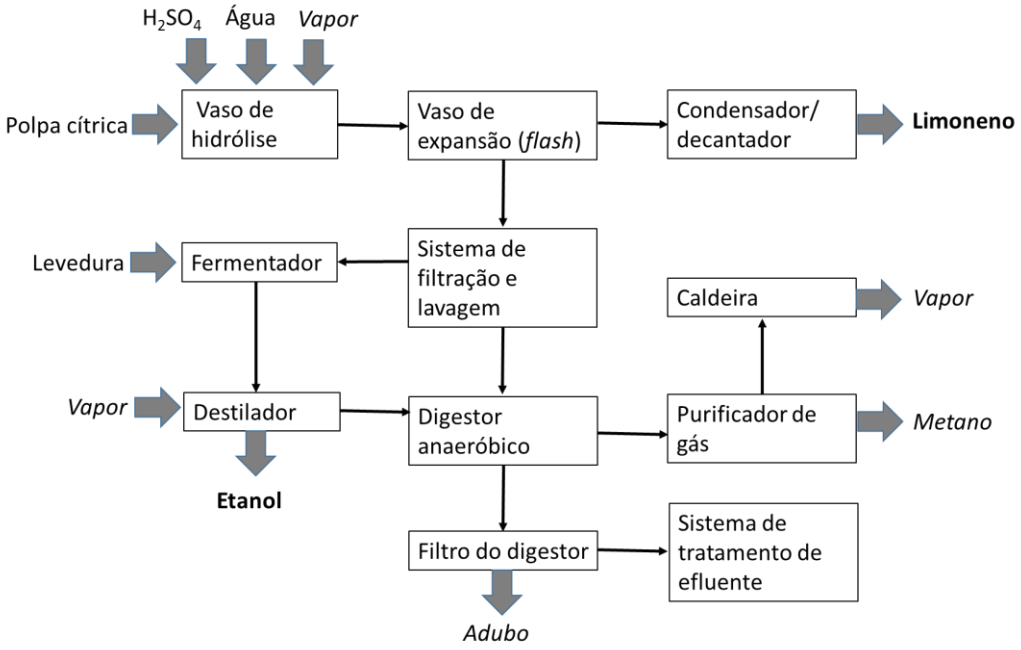

Figura 3. Descrição do processo da biorrefinaria proposta por LOHRASBI et al. (2010)

RIVAS-CANTU, JONES e MILLS (2013) avaliaram as principais dificuldades enfrentadas pelas biorrefinarias de polpa cítrica, como as propostas por LOHRASBI *et al* (2010) e LOPEZ, LI e THOMPSON (2010). Os maiores problemas encontrados foram: (a) o estado atual da tecnologia referente à produção de açúcares fermentescíveis do hidrolisado de polpa cítrica e (b) o rendimento de etanol por *S. cerevisiae* a partir deste hidrolisado. Considerando isso, eles calcularam o tamanho de indústria necessário para tornar o processo viável: a planta teria que ter capacidade para processar 3,3 milhões de toneladas de polpa cítrica fresca, que é uma quantidade tão grande quanto toda a produção de polpa cítrica do estado da Flórida (EUA). O autor recomendou, então, a produção paralela de outros produtos de maior valor agregado, como ácido D-galacturônico e flavonoides.

O projeto de cooperação AKA/CNPq também visa à produção de uma diversidade de produtos numa biorrefinaria de polpa cítrica. Essencialmente, a construção de linhagens de leveduras recombinantes que consigam aproveitar melhor o hidrolisado de polpa cítrica para a produção de etanol também seria imprescindível. O leque de produtos da biorrefinaria proposta pelo projeto de cooperação AKA/CNPq (Fig. 4) se amplia para além dos que foram propostos por LOHRASBI *et al* (2010) e LOPEZ, LI e THOMPSON (2010), incluindo produtos de alto valor agregado, como o ácido D-galacturônico, o ácido ascórbico, o ácido L-galactônico e o ácido *meso*-galactárico, que podem vir a ser intermediários de síntese química (*platform chemicals*) estratégicos.

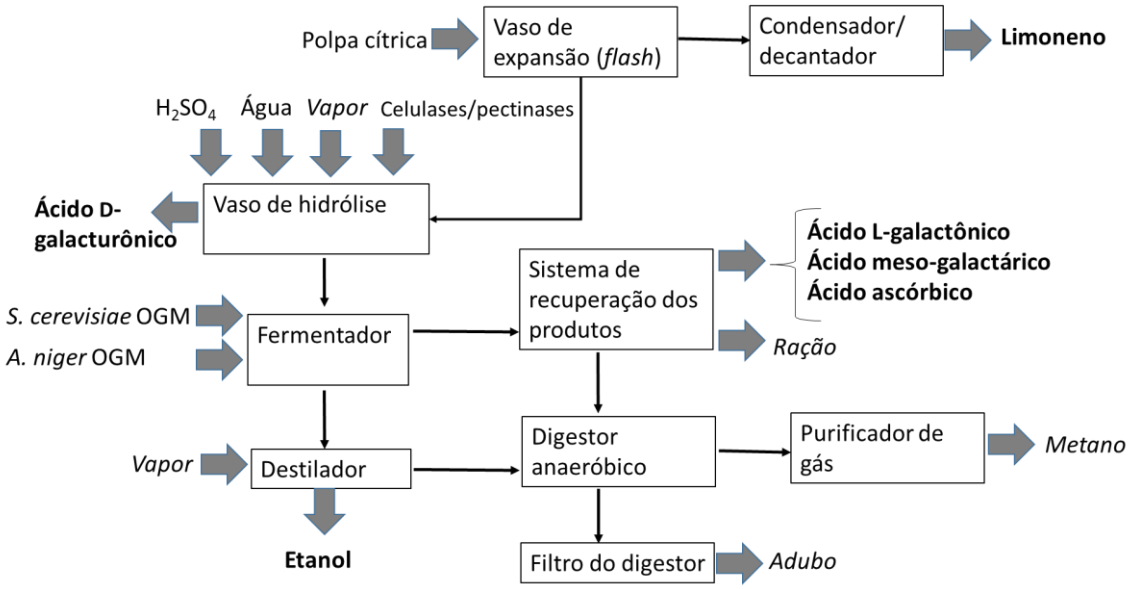

Figura 4. Descrição do processo da biorrefinaria proposta pelo projeto de cooperação AKA/CNPq, adaptado das ideias originais de LOPEZ, LI e THOMPSON (2010) e LOHRASBI et al. (2010)

2.2 Descrição dos produtos da biorrefinaria proposta pelo projeto AKA/CNPq

Como já mencionado, a via redutiva do catabolismo do ácido D-galacturônico foi elucidada pelo grupo de biologia sintética da VTT (HILDITCH *et al.* 2007). Com base na descoberta desta via, o grupo de biologia sintética da VTT observou a oportunidade de utilizar as técnicas de engenharia metabólica para a construção de linhagens de micro-organismos recombinantes para a produção dos materiais

propostos pela biorrefinaria. A seguir, são detalhados, primeiro, a via em si, e segundo, os produtos propostos para a biorrefinaria do projeto de cooperação e as estratégias de engenharia metabólica utilizada para obtê-los.

A via redutiva do catabolismo do ácido D-galacturônico consiste em 4 enzimas, com duas etapas de redução (Fig. 5). A primeira enzima da via, a D-galacturonato redutase (EC 1.1.1.365), codificada pelo gene *gaaA* em *A. niger*, converte o ácido D-galacturônico a L-galactonato, utilizando um NADH. A L-galactonato desidratase (EC 4.2.1.146), codificada pelo gene *gaaB* em *A. niger* e pelo gene *lgd1* em *T. reesei*, converte esse composto a 2-ceto-3-deoxi-galactonato. A 2-ceto-3-deoxi-galactonato aldolase (EC 4.1.2.B7), codificada pelo gene *gaaC* em *A. niger*, faz uma clivagem da ligação entre os carbonos 3 e 4, formando um piruvato e um L-gliceraldeído. Nesses micro-organismos, o piruvato entra no metabolismo pelo ciclo do ácido cítrico, e o L-gliceraldeído é convertido a glicerol pela gliceraldeído redutase (EC 1.1.1.372), codificada pelo gene *gaaD* em *A. niger*, gastando, para isso, um NADPH.

Os produtos propostos estão listados abaixo:

- Ácido D-galacturônico
- Ácido *meso*-galactárico
- Ácido L-galactônico
- Ácido ascórbico
- Etanol de ácido D-galacturônico

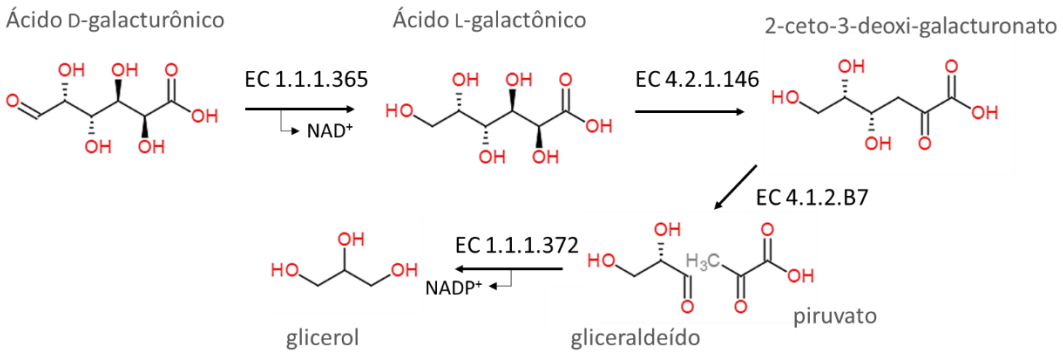

Figura 5. Via redutiva do catabolismo do ácido D-galacturônico. Adaptado de HILDITCH et al. (2007)

Ácido D-galacturônico: O principal açúcar da pectina é o ácido D-galacturônico, que corresponde a 75-80% (m/m) da pectina cítrica (SIGMA, 2011). A característica ácida deste açúcar (pK_a 3,5) torna simples a sua recuperação a partir do meio de hidrólise com a técnica de eletrodiálise (MOLNAR *et al.,* 2009). Pode ser comercializado diretamente para a utilização na indústria alimentícia, como agente acidificante, e na indústria química como agente tensoativo. Este açúcar também pode ser transformado quimicamente ou biotransformado através de técnicas de engenharia metabólica em substâncias de maior valor agregado, como o ácido múcico (ácido *meso*-galactárico), o ácido ascórbico (Vitamina C) e o ácido L-galactônico (RICHARD e HILDITCH, 2009; MOJZITA *et al*., 2010), ou fermentado a etanol por leveduras recombinantes (HUISJES *et al.* 2012). Na tentativa de produzir ácido D-galacturônico como produto final, o grupo de pesquisa da VTT tem concentrado esforços para produzir um *A. niger* recombinante capaz de crescer em polpa cítrica e, ao mesmo tempo, liberar as pectinases para hidrolisar a polpa cítrica, mas sem utilizar este açúcar. Isso simplificaria o processo da biorrefinaria proposta. A deleção do gene da primeira enzima da via em *A. niger*, *gaaA*, no entanto, não surtiu o efeito esperado de impedir o consumo de ácido D-galacturônico pelo fungo (Fig. 5). O grupo da VTT ainda fez uma pesquisa no genoma, mas não foram encontrados genes das enzimas putativas de *A. niger* que poderiam estar substituindo a enzima EC 1.1.1.365, cujo gene foi deletado (MOTTER *et al.*, 2013). Este estudo foi feito pela aluna brasileira de iniciação científica na VTT (Espoo) em seu estágio pelo Ciências Sem Fronteiras.

Ácido *meso*-galactárico: O ácido *meso*-galactárico, também conhecido como ácido múcico, tem aplicações como fermento químico ou como substituinte do ácido tartárico em refrigerantes. É, também, um material de partida para síntese química: pode ser convertido no ácido 2,5- furandicarboxílico (FDCA), um dos doze compostos químicos de maior valor agregado produzidos a partir de biomassa (*Top Value-Added Chemicals from Biomass*) (EERE, 2004). O FDCA poderá substituir o ácido tereftálico como monômero para a produção de poliésteres (especificamente o PET). A síntese química do ácido múcico não é atrativa, pois envolve a oxidação da D-galactose com ácido nítrico, produzindo efluentes tóxicos (RICHARD e HILDITCH, 2009). A metodologia do processo de bio-conversão de ácido D-galacturônico a ácido múcico

por *A. niger* foi descrita pelo grupo de biologia sintética da VTT (MOJZITA *et al.* 2010) (Fig. 6). As principais modificações gênicas utilizadas para produzir este composto foram a deleção do gene de EC 1.1.1.365 (*gaaA*) e inserção do gene *udh*, que corresponde à enzima EC 1.1.1.203 (uronato desidrogenase, *Agrobacterium tumefaciens*).

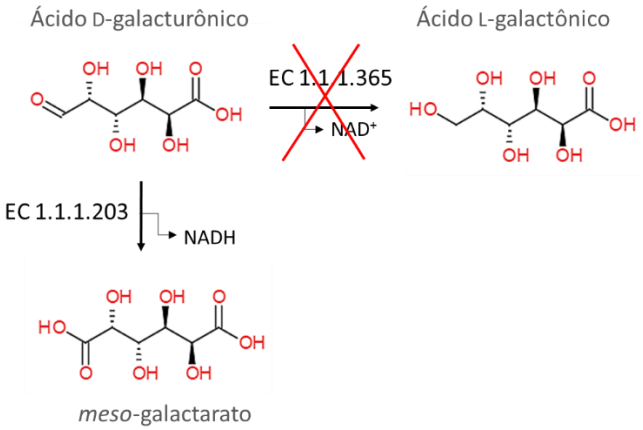

Figura 6. Conversão de ácido D-galacturônico a ácido múcico por uma cepa geneticamente modificada de *Aspergillus niger*

Ácido L-galactônico: Este açúcar ainda não é muito utilizado na indústria, devido ao seu alto custo de produção. No entanto, sua utilização como insumo industrial pode ser considerada, caso seu custo de produção seja diminuído graças às biorrefinarias baseadas em pectina (MOJZITA *et al.*, 2010). Este ácido tem propriedades similares às do ácido D-glucônico, que é atualmente aplicado como aditivo em cosméticos, em alimentos como acidulante, em produtos de limpeza (devido as suas propriedades como agente quelante) e como um aditivo na formulação de concreto (retardador) (RICHARD e HILDITCH, 2009). A metodologia do processo de bioconversão de ácido D-galacturônico a ácido L-galactônico por *A. niger* foi desenvolvida no âmbito do projeto de cooperação (KUIVANEN *et al.* 2014), e consiste na deleção do gene *gaaB*, que codifica a enzima EC 4.2.1.146, do genoma de *A. niger* (Fig. 5). Parte deste trabalho foi feito no Laboratório de Tecnologia Fermentativa e Enzimática pelo pesquisador Hugo Dantas da VTT em seu estágio no Brasil.

O ácido ascórbico: O ácido ascórbico (Vitamina C) é usado principalmente como suplemento vitamínico e como antioxidante na indústria alimentícia. Tem mercado aproximado de 80 mil toneladas/ano, o equivalente a 600 milhões de dólares. Industrialmente, é obtido quimicamente a partir da glucose, mas a produção por uma via biossintética, utilizando ácido D-galacturônico como substrato inicial, foi descrita por KUIVANEN, PENTILLA e RICHARD (2015), no âmbito do projeto de cooperação. As modificações genéticas feitas em *A. niger* para produzir o ácido L-ascórbico foram as seguintes: a deleção do gene *gaaB*, que codifica a enzima EC 4.2.1.146, do genoma de *A. niger* e a inserção de dois genes de plantas *Alase* (*Euglena gracilis*) e *galDH* (*Malpighia glabra*) que codificam para as enzimas EC 3.1.1.17 e EC 1.3.2.3, respectivamente (Fig. 7).

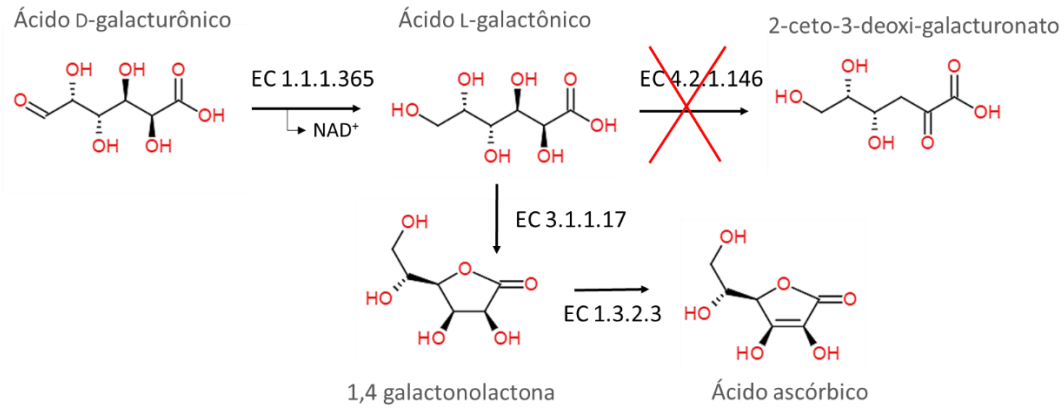

Figura 7. Conversão de ácido D-galacturônico a ácido L-ascórbico por uma cepa geneticamente modificada de *Aspergillus niger*

Etanol a partir de ácido D-galacturônico: O etanol tem demanda em ascensão no Brasil e um grande potencial para ser o principal produto de uma biorrefinaria de polpa cítrica. O país já foi um grande expoente na produção deste biocombustível nos anos 70, e, mais recentemente, no início do século XXI, com a certeza da escassez e elevação nos preços dos combustíveis derivados do petróleo, houve um novo interesse na expansão da indústria do etanol no Brasil. Atualmente, há uma mistura obrigatória de etanol anidro à gasolina de 27%. Além disso, há também um crescente aumento da frota de carros *flex*, que este ano já passou de 50% da frota e nas exportações de etanol para os Estados Unidos, que já supera 3 bilhões de litros

(VALOR ECONÔMICO, janeiro 2013). Para a safra de 2015, está prevista a produção de 30 bilhões de litros de etanol. A polpa cítrica é uma fonte alternativa à cana-de-açúcar e tem potencial para contribuir no aumento da produção de etanol no Brasil. No entanto, 18% (m/m) da polpa cítrica–é composto de ácido D-galacturônico, um açúcar que não é fermentescível por *Saccharomyces cerevisiae*, o micro-organismo de escolha para a produção de etanol em usinas. Portanto, o estabelecimento de uma tecnologia de conversão de ácido D-galacturônico a etanol ainda exige o desenvolvimento de cepas geneticamente modificadas que sejam capazes de utilizar esse açúcar.

2.3 Desafios das biorrefinarias de polpa cítrica

Conforme mencionado anteriormente, já foram desenvolvidas estratégias para a produção de ácido L-galactônico, de ácido *meso*-galactárico e de ácido ascórbico a partir de ácido D-galacturônico no âmbito do projeto de cooperação. Os principais desafios que ainda cercam os processos da biorrefinaria de polpa cítrica proposta pelo projeto de cooperação AKA/CNPq são: (1) a hidrólise da pectina, (2) a produção de pectinases de baixo custo e (3) a produção de etanol também a partir do ácido D-galacturônico. Esses desafios serão abordados com detalhe, a seguir.

2.3.1 Hidrólise da pectina

As pectinas compreendem um grupo de polissacarídeos ácidos, formados quase que inteiramente por uma cadeia principal de resíduos de ácido D-galacturônico, unidos por ligações glicosídicas α(1→4). No entanto, as pectinas podem apresentar grande diversidade em sua estrutura molecular. Os resíduos de ácido D-galacturônico podem estar metilados ou acetilados, podem ser intercalados por ligações com resíduos de ramnose (ligados em α(1→2)). Além disso, a cadeia principal pode conter ramificações de polímeros de outros açúcares como a arabinose, galactose, xilose, e ainda pode conter açúcares raros, como a apiose. Além disso, as pectinas podem ser classificadas como homogalacturonanas (HG),

ramnogalacturonanas I (RG-I), ramnogalacturonanas II (RG-II), dependendo da sua constituição e estrutura primária (Fig. 8).

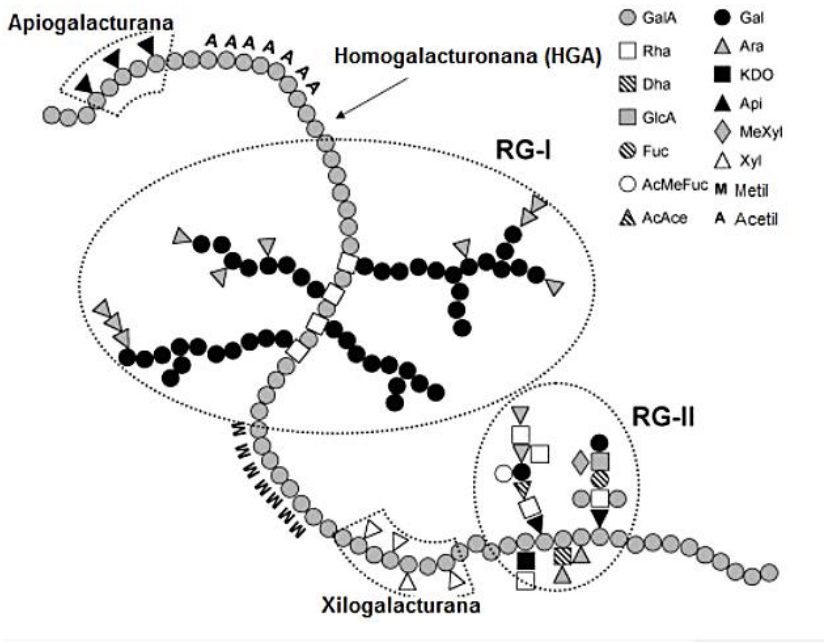

Figura 8. Modelo de estrutura para a molécula da pectina. Fonte: PÉREZ, RODRÍGUEZ-CARVAJAL e DOCO (2003). Legenda: RG-I: ramnogalacturonana tipo I; RG-II:Ramnogalacturana tipo II; GalA: ácido D-galacturônico; Rha: ramnose; Dha: ácido 3-desoxi-D-lixo-2-heptulosárico; GlcA: ácido glucurônico; Fuc: fucose; AcMeFuc: acetilmetilfucose; AcAce: ácido acérico; Gal: galactose; Ara: arabinose; KDO: ácido-2-ceto-3-deoxi-D-octulosônico; Api: apiose; MeXyl:metil-xilose; Xyl: xilose. M: metil, A: acetil.

A pectina pode ser hidrolisada a seus açúcares constituintes com tratamento com ácido mineral diluído, em geral ácido clorídrico ou sulfúrico, em pH 1 a 2, e numa faixa de temperatura que vai de 100 a 150 °C.

As condições satisfatórias para a hidrólise ácida de um substrato polissacarídico, como a pectina, podem ser encontradas por intermédio de curvas apropriadas, nas quais são fatores variáveis a concentração de ácido da solução, a temperatura e a duração da hidrólise. É no pico máximo de produção de açúcares redutores que devem ser fixadas as condições de hidrólise, pois, além desse ponto, há mais destruição dos açúcares já formados que liberação de mais unidades de açúcar pela hidrólise (SAEMAN *et al.,* 1954). Em outras palavras, o aumento

excessivo da concentração de ácido, do tempo ou da temperatura, para elevar a velocidade de hidrólise da cadeia poligalacturônica, destrói os monômeros de açúcar já liberados. É essa característica dos processos de hidrólise ácida que o tornam inadequados para a obtenção máxima dos açúcares livres da pectina.

A estrutura complexa da pectina tem uma implicação para a hidrólise química deste polissacarídeo porque cada ligação, e cada açúcar, tem uma suscetibilidade diferente ao tratamento ácido (BLAKE e RICHARDS, 1968). Os açúcares neutros têm a estrutura mais lábil frente ao ataque ácido após liberados, em especial a arabinose, que é um açúcar abundante em pectinas de resíduos cítricos. As ligações α(1→4) entre os resíduos de ácido D-galacturônico da região de homogalacturanana são as mais difíceis de serem quebradas pelo ataque ácido, devido aos efeitos indutivos e conformacionais causados pelos grupamentos carboxílicos próximos (BLAKE e RICHARDS, 1968). Portanto, as condições químicas necessárias para a hidrólise total das homogalacturonanas são tão agressivas que os açúcares neutros que são liberados nos primeiros momentos do ataque ácido são decompostos, formando lactonas de maneira irreversível.

A melhor estratégia para obter ácido D-galacturônico a partir da pectina de polpa cítrica envolve duas etapas. A primeira etapa é uma pré-hidrólise com ácido mineral diluído que degrada a hemicelulose presente nas cascas, auxiliando a liberação da pectina quase intacta. Esta etapa, que é usada comercialmente na recuperação de pectina para uso na indústria alimentícia (MAY, 1990), produz um resíduo insolúvel, muito rico em celulose, e pectina solubilizada, misturada com monossacarídeos e oligossacarídeos extraídos da casca da laranja. A segunda etapa envolve a hidrólise enzimática da pectina com pectinases.

Pectinases são um amplo conjunto de enzimas responsáveis pela solubilização, desesterificação e despolimerização da complexa estrutura da pectina nativa (SCHUBERT, 1952). Em conjunto, estas enzimas levam à liberação dos açúcares redutores da pectina. Diferentemente da hidrólise ácida, a hidrólise enzimática não decompõe os açúcares livres. Dentre essas enzimas se destacam as despolimerases, que são as principais pectinases produzidas por micro-organismos e catalisam a quebra das ligações glicosídicas α (1→4) na cadeia de ácido poligalacturônico. Podem ser do tipo pectina liase (PEL, EC 4.2.2.10); ou pectato-liase

(PLY, EC 4.2.2.9) (Fig 9A), que clivam a cadeia poligalacturônica através de β-eliminação, ou do tipo poligalacturonase (PGA, EC 3.2.1.15; XPG, EC 3.2.1.67) (Fig 9B), que clivam a cadeia poligalacturônica através de hidrólise (SAKAI, 1992; ALKORTA, GARBISU e SERRA,1997; de VRIES *et al.,* 2002).

Também contribuem para a formação de açúcares redutores as enzimas do tipo metil ou acetil-esterases (PME, EC 3.1.1.11; AE, EC 3.1.1.6) (Fig 9C), pois desesterificam a pectina, facilitando a ação das poligalacturonases e pectato-liases. Endo-ramnogalacturonana hidrolases (EC 3.2.1.) e ramnogalacturonana liases (EC 4.2.2.), que atuam sobre as ligações glicosídicas entre a ramnose e o ácido galacturônico presentes em certas regiões da cadeia péctica, também liberam pontas redutoras. Além disso, enzimas acessórias, como as endo e exo-galactanases (EC 3.2.1.89; EC 3.2.1.145), α e β-galactosidades (EC 3.2.1.22; EC 3.2.1.23), endo e exo-arabinases (EC 3.2.1.99; EC 3.2.1.), e α-L-arabinofuranidades (EC 3.2.1.55), também contribuem para a formação de açúcares redutores pela hidrólise das ligações entre os resíduos nas cadeias laterais de açúcares neutros da pectina (de VRIES *et al.* 2002).

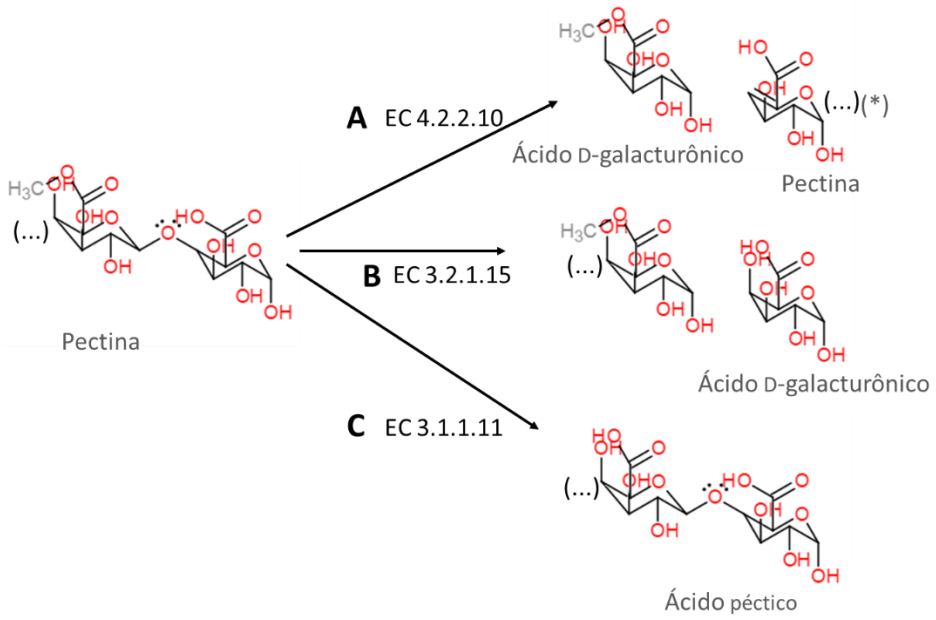

Figura 9. Enzimas pectinolíticas. A) pectato/pectina-liase. B) Poligalacturonases. C) Esterases.
* Oligossacarídeo com grupamento 4-deoxi-α-D-galacto-4-enurosil na ponta não redutora.

A aplicação de pectinases em biorrefinarias, no entanto, é dificultada pelo alto custo das enzimas e pela baixa velocidade da reação de despolimerização. No passado, esta lentidão foi atribuída à inibição pelo ácido D-galacturônico (BACIU e JÖRDENING, 2004; BÉLAFI-BAKÓ *et al.,* 2007; KHAMSEH e MICCIO, 2012). No entanto, a inibição pelo ácido D-galacturônico foi abordada somente de maneira superficial na literatura e não se pode afirmar que é a única causa para a lentidão da reação.

O primeiro artigo que faz parte desta tese (Capítulo 2) mostrou que existia uma desaceleração abrupta na liberação de açúcares redutores nos primeiros minutos da reação, um fenômeno que não tinha sido relatado anteriormente. Esta descoberta deu base para o trabalho posterior de FARIAS (2014). FARIAS (2014) estudou se o que causava a desaceleração inicial da velocidade das pectinases era mesmo a inibição pelo produto, ou se era um fenômeno de *burst*, que é o que causa a desaceleração na hidrólise de celulose por celulases, ou, ainda, se era a depleção rápida de substratos mais facilmente degradados. Por fim, ela levantou a hipótese de que era a depleção do substrato preferencial das endo-pectinases, os polissacarídeos de cadeia longa, que leva a essa desaceleração inicial. A Figura 10 exemplifica a explicação dada pela autora.

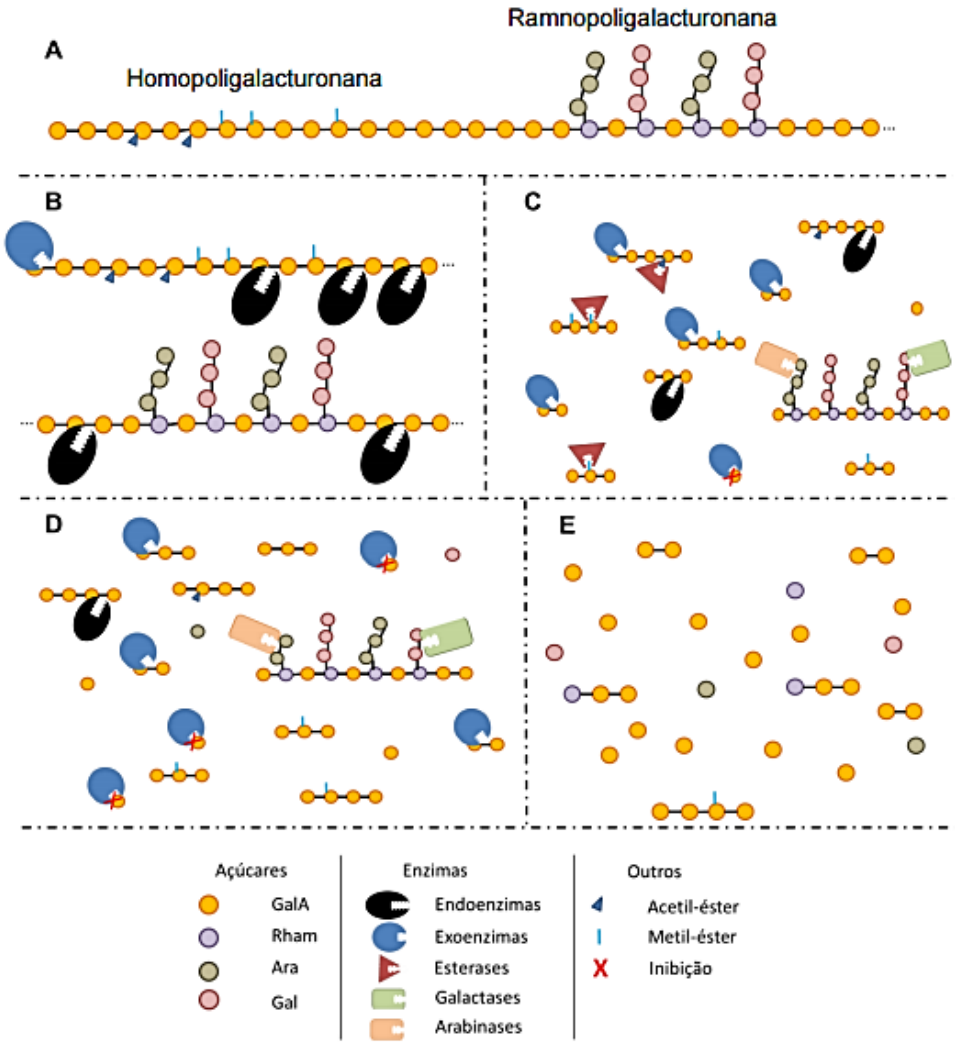

Figura 10. Hipótese sobre o modo de ação das pectinases sobre a pectina.
(A) Estrutura da pectina simplificada, com destaque para as regiões de homopoligalacturonana e ramnogalacturonana. (B) Ação das enzimas nos primeiros minutos de degradação, antes da desaceleração da velocidade de reação, onde há uma maior atividade das endoenzimas no substrato. (C) Meio reacional no momento em que ocorre a desaceleração inicial da velocidade de reação, evidenciando a diminuição de substrato para as endoenzimas, e maior ação das exoenzimas e enzimas auxiliares (ramnogalacturonases, arabinases, galactases, xilanases e esterases). (D) Segunda etapa da reação enzimática, onde ocorre uma desaceleração prolongada da velocidade de despolimerização, devido à limitação da ação das exoenzimas pela dependência da catalise promovida pelas enzimas auxiliares e pela inibição causada pelo produto. (E) Meio reacional após 24 horas de reação, com alguns oligômeros recalcitrantes restantes e muitos monossacarídeos liberados. Fonte: FARIAS (2014).

2.3.2 Produção de pectinases por fermentação no estado sólido

Pectinases são produzidas em escala comercial e já têm um mercado estabelecido nas indústrias de alimentos, bebidas e rações. São utilizadas principalmente na extração de óleos vegetais, na clarificação de sucos e para

melhorar as características organolépticas dos vinhos (KASHYAP *et al.*, 2001). Também podem ser utilizadas para melhorar as qualidades nutricionais de rações de aves (JAYANE, SAXENA e GUPTA, 2005). No entanto, as pectinases atualmente disponíveis têm um custo proibitivo para a aplicação em biorrefinarias de polpa cítrica, já que é necessário utilizar uma quantidade relativamente maior de enzima no processo de hidrólise. De fato, a hidrólise da pectina é um processo central em biorrefinarias de polpa cítrica, como as propostas por LOHRASBI *et al.,* (2010) e pelo projeto de cooperação. Portanto, é fundamental para a viabilidade econômica dessas indústrias que pectinases de baixo custo e robustas estejam disponíveis.

As pectinases comerciais, como a maioria das enzimas comerciais, são produzidas por fermentação submersa (FS). Por outro lado, a produção de pectinases por fermentação no estado sólido (FES) pode ser considerada para a diminuição do seu custo. As principais vantagens da FES em relação à FS são a maior produtividade volumétrica, o que implica em menores volumes dos biorreatores, menores custos de *downstream*, e a significativa redução do volume de efluentes, devido ao meio mais concentrado. Além disso, a FES frequentemente utiliza substratos abundantes e de baixo custo, tais como os resíduos agroindustriais, o que diminui o custo do processo (PANDEY, SOCCOL e MITCHELL, 2000). Diversos estudos comparativos de produção de pectinases por FES e FS identificaram que a FES resulta em maior produtividade de pectinases, ou seja, um maior número de unidades de atividade produzidas por hora por massa de substrato utilizado (TREJO-HERNANDEZ *et al*., 1991; SOLIS-PEREYRA *et al*., 1993; MALDONADO e SAAD, 1998; DÍAZ-GODÍNEZ *et al*., 2001). A produção de pectinases em FES também não necessita de indução por produtos de degradação da pectina (VINIEGRA-GONZALEZ e FAVELA-TORRES, 2006), e não sofre repressão catabólica (SOLIS-PEREYRA *et al*., 1993, 1996).

A produção de pectinases em escala de bancada por FES, sobretudo em processos feitos com fungos filamentosos dos gêneros *Aspergillus* e *Penicillium*, tem sido extensamente estudada nos últimos 20 anos (ANTIER *et al*., 1993; KAVITHA e UMESH-KUMAR, 2000; FAVELA-TORRES *et al*., 2006; TEIXEIRA *et. al.,* 2011). No entanto, apesar das vantagens previamente citadas, a viabilização da produção industrial de pectinases em FES possui alguns desafios intrínsecos a este sistema: a tecnologia ainda não está bem estabelecida e existem poucos estudos publicados

com produção de metabólitos por FES em escala piloto. No caso específico da produção de pectinases, existe apenas 2 trabalhos em escala maior que 2 Kg (HUERTA *et al.,* 1994; HE e CHEN, 2013).

A FES é caracterizada pelo crescimento do microrganismo em um substrato sólido úmido, mas na ausência de água livre. Isso leva a dificuldades diferentes no escalonamento de processos: enquanto que a principal dificuldade de escalonamento da fermentação líquida é a difusão de oxigênio, na FES esses problemas estão mais relacionados com a remoção do calor metabólico do leito de partículas sólidas contido no biorreator. Se o calor não é removido adequadamente, a temperatura do leito aumenta, podendo chegar a valores que podem causar a interrupção no crescimento do micro-organismo e a desnaturação das enzimas produzidas (MITCHELL, VON MEIEN e KRIEGER, 2003). Enquanto que, nos biorreatores de FS, camisas, serpentinas e a agitação constante do líquido garantem uma temperatura estável em todos os pontos da fermentação, em biorreatores de FES esses acessórios não são efetivos para o resfriamento do leito.

Os diversos biorreatores de FES contam com estratégias diferentes para manter a temperatura do leito num nível aceitável. Os processos em biorreatores de bandeja, por exemplo, limitam bastante a altura do leito, para poucos centímetros de altura. O escalonamento deste tipo de biorreator, portanto, envolve a utilização de um maior número de bandejas. Essa estratégia, no entanto, pode encarecer o processo porque a razão entre volume útil e volume total fica pequena, e tipicamente, também aumenta o custo de mão de obra, já que são necessários operários para manipular as bandejas. Outro tipo de biorreator de FES é o tambor rotativo. Ele tenta minimizar as dificuldades de transferência de massa pela agitação constante do leito. Esse tipo de biorreator tem a desvantagem de quebrar as redes de micélios formadas por fungos filamentosos, que é o tipo de micro-organismo geralmente utilizado para a produção de pectinases. Essa quebra do micélio pode ter um efeito deletério para o crescimento do micro-organismo e para a produção de produto.

Os biorreatores de leito fixo são equipamentos que recebem aeração forçada continuamente pela parte de baixo do leito e que, apesar de poderem ser agitados de modo intermitente, ficam sem agitação durante a maior parte do tempo. A aeração forçada é responsável por remover o calor metabólico gerado pelo micro-organismo

em crescimento, além de fornecer O_2 ao leito de fermentação. Entretanto, a aeração forçada pode provocar a secagem do leito. Mesmo que o ar entre no leito saturado com água, conforme o ar atravessa o leito, a transferência de calor do leito eleva sua temperatura e, consequentemente, aumenta sua capacidade de carregar água (VON MEIEN e MITCHELL, 2002). Além disso, há um limite para a altura do leito de biorreatores de leito fixo. Quanto maior é o tamanho do leito, maior será a distância que o ar terá que percorrer dentro do leito, o que acarreta uma maior temperatura na região do topo do leito. Portanto, o leito deve ter uma altura suficientemente baixa para fazer com que temperaturas deletérias não sejam alcançadas no topo do leito (MITCHELL *et al.,* 1999).

Um problema frequente em biorreatores de leito fixo, e que precisa ser evitado, é a formação de caminhos preferenciais para o fluxo de ar. Esses caminhos preferenciais são formados devido a fissuras no leito, ou encolhimento do leito de fermentação, que acaba afastando-o das paredes do biorreator. Essa formação de caminhos preferenciais faz com que o ar passe ao redor do leito, e não através dele, causando a elevação da temperatura no interior do leito. Esses caminhos preferenciais são criados principalmente em processos que utilizam fungos filamentosos, já que, durante o seu crescimento, eles agregam as partículas de substrato, provocando a compactação do leito. O leito compactado acaba encolhendo, afastando-se das paredes (Fig. 11 A-C).

Em um estudo anterior do nosso grupo (PITOL *et al.,* 2016) foi avaliada a produção de pectinases em escala piloto utilizando para isso o fungo filamentoso *Aspergillus niger* e crescendo em uma mistura de 90:10 (m/m, massa seca) de farelo de trigo e bagaço de cana. Neste estudo foram observados sérios problemas de compactação do leito, com a consequente formação de caminhos preferenciais para o fluxo de ar e superaquecimento do leito. O segundo artigo desta tese (Capítulo 3) apresenta um estudo que solucionou esses problemas ao usar significativamente mais bagaço de cana como agente de volume. Este trabalho envolveu a utilização do fungo filamentoso *Aspergillus oryzae* crescendo em uma mistura de 51,6:48,4 (m/m, massa seca) de polpa cítrica e bagaço de cana. Neste meio, os problemas de compactação foram totalmente superados e, consequentemente, foi possível controlar a temperatura num intervalo que foi favorável ao crescimento do micro-organismo.

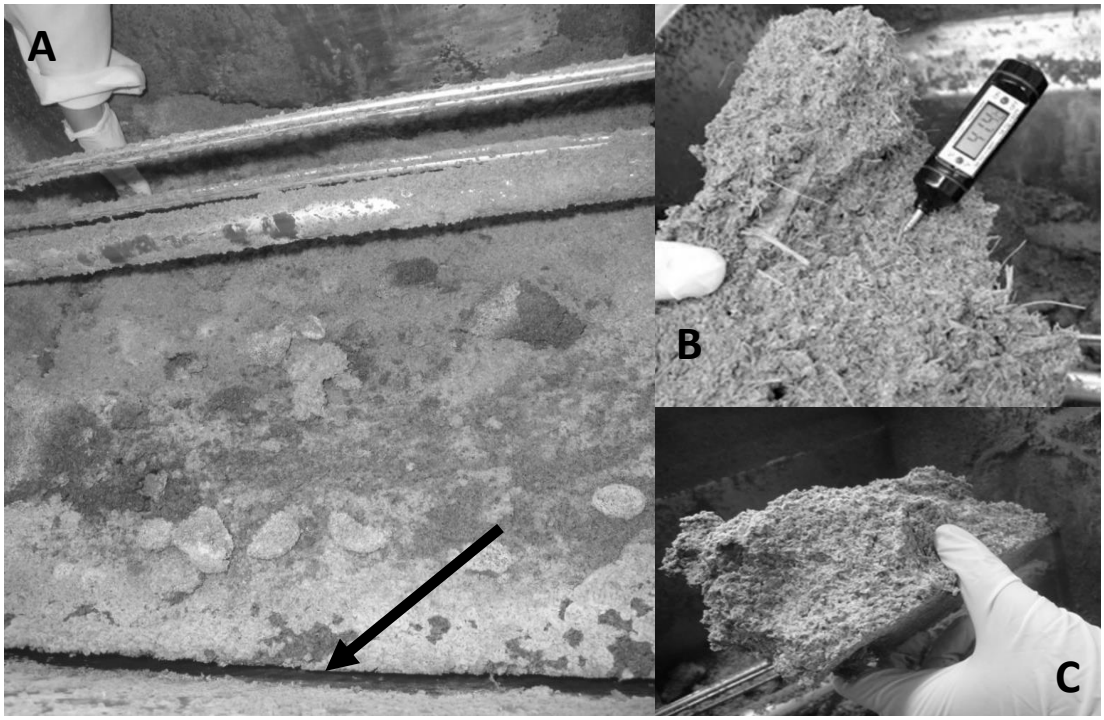

Figura 11. Fermentação no estado sólido em biorreator de leito fixo. A) Formação de caminhos preferenciais próximo à parede de um biorreator de leito fixo. B) O termômetro mostra cerca de 10°C a mais no interior do sólido fermentado que a temperatura ótima fermentação. C) Bloco de sólido fermentado compactado que se formou no interior do biorreator.

2.3.3 Produção de etanol a partir do hidrolisado de polpa cítrica

Após hidrólise enzimática com celulases e pectinases, o hidrolisado contém açúcares fermentescíveis por *S. cerevisiae* como a glucose, frutose, galactose e sacarose, e que representam cerca de 47% do hidrolisado (Tabela 1) (GROHMANN e BALDWIN, 1992). Os demais açúcares, principalmente a arabinose que corresponde a cerca de 7% do hidrolisado, e o ácido D-galacturônico, que corresponde a 18%, não são fermentescíveis por esta levedura. Em outras palavras, é uma porção significativa do hidrolisado que não pode ser convertido a etanol por *S. cerevisiae*.

Tabela 1. Conteúdo de açúcares da polpa cítrica após hidrólise com celulases e pectinases

Açúcares	**% m/m (base seca)**
Glucose	24
Ácido D-galacturônico	18
Frutose	14
Arabinose	7
Galactose	5
Sacarose	4

Nota: Adaptado de GROHMANN e BALDWIN, 1992

Potencialmente, tanto leveduras quanto bactérias podem ser modificadas geneticamente para a conversão de ácido D-galacturônico a etanol. A conversão do ácido D-galacturônico em etanol em *Escherichia coli* foi feita com sucesso por DORAN *et al*. (2000). *E. coli* possui a via bacteriana (ou via da isomerase) do catabolismo do ácido D-galacturônico, mas produz etanol em fermentação em pouca quantidade devido à seu metabolismo de ácidos mistos. Por esta razão, DORAN *et al*. (2000) clonaram dois genes de *Zymomonas mobilis*, o gene da álcool desidrogenase e o gene da piruvato descarboxilase, em *E. coli*. A cepa resultante, *E. coli* KO11, conseguiu converter ácido D-galacturônico, quando fornecido como a única fonte de carbono, a etanol, atingindo um rendimento de 0,19 g etanol/g açúcar e concentração máxima de 3,86 g de etanol/L. No entanto, essa produtividade não é suficientemente alta para a escala comercial de produção.

A produção de etanol por *S. cerevisiae*, por outro lado, já é um processo de alta produtividade e robustez. Ao contrário da maioria das bactérias, a *S. cerevisiae*, é mais resistente às condições não ideais de produção que ocorrem na escala industrial como, por exemplo, o baixo pH e a presença de contaminantes, bacteriófagos e inibidores. Além disso, tolera uma pressão osmótica muito mais elevada e uma maior quantidade de etanol, o que possibilita um meio de cultivo mais concentrado. Esse meio mais concentrado impacta bastante nos custos de produção das usinas de álcool, porque os biorreatores podem ser menores e a quantidade de água que entra no processo de destilação também é menor (VAN MARIS *et al.*, 2006).

Em face disso, a clonagem de uma via heteróloga de catabolismo do ácido D-galacturônico em *S. cerevisiae*, em vez da clonagem das enzimas alcoogênicas em bactérias, seria a estratégia com maior possibilidade de sucesso na indústria e poderia ajudar a superar a produção de etanol esperada a partir da hidrólise da polpa cítrica. O terceiro artigo desta tese trata desta clonagem. Para tanto, foi considerado com cuidado quais genes envolvidos no catabolismo de ácido D-galacturônico seriam utilizados.

Além da via redutiva, elucidada para *A. niger* e *T reseei*, e já descrita anteriormente nesta revisão, existem ainda outras duas vias conhecidas de catabolismo de ácido D-galacturônico, a via bacteriana (da isomerase) e a via oxidativa.

A via bacteriana foi demonstrada em *E. coli* e consiste em cinco enzimas que convertem o ácido D-galacturônico a piruvato e D-gliceraldeído-3-fosfato (Fig. 12). A primeira enzima desta via, a uronato isomerase (EC 5.3.1.12) converte o ácido D-galacturônico a D-tagaturonato. Em seguida, a D-tagaturonato redutase (EC 1.1.1.58) reduz o D-tagaturonato a D-altronato, utilizando, para isso, um NADH. A próxima enzima da via, a altronato desidratase (EC 4.2.1.7) converte o D-altronato a 2-ceto-3-deoxi-D-gluconato. A 2-ceto-3-deoxi-D-gluconato quinase (EC 2.7.1.45) adiciona um fosfato, com o gasto de um ATP, formando 2-ceto-3-deoxi-fosfogluconato. Por último, a 2-ceto-3-deoxi-fosfogluconato aldolase (EC 4.1.2.14) converte esse composto a piruvato e D-gliceraldeído-3-fosfato.

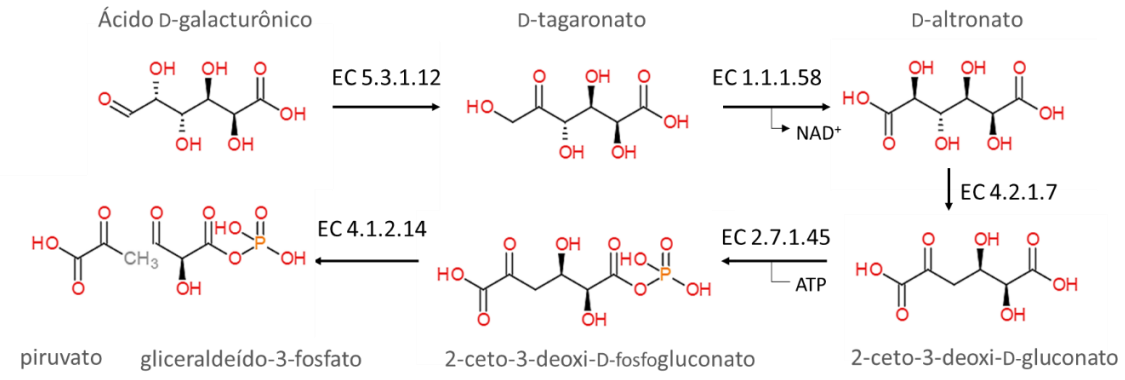

Figura 12. A via bacteriana de catabolismo de ácido D-galacturônico

A via oxidativa é uma via bacteriana alternativa, demonstrada em *Agrobacterium tumefaciens* e em algumas espécies do gênero *Pseudomonas* (Fig. 13). Essa via consiste em quatro enzimas e converte o ácido D-galacturônico a 2-ceto-glutarato em duas etapas de oxidação, com o gasto de 2 NAD^+, e duas etapas de desidratação. A primeira enzima da via, a D-galacturonato desidrogenase (EC 1.1.1.203) converte o ácido D-galacturônico a *meso*-galactarato, com o consumo de um NAD^+. Em seguida, ocorrem duas desidratações: primeiramente, a D-galactarato desidratase (EC 4.2.1.42) converte o *meso*-galactarato a 2-deoxi-2-ceto-L-treo-hexarato e, depois, a 5-dehidro-4-deoxi-glucarato desidratase (EC 4.2.1.41) converte o 2-deoxi-2-ceto-L-treo-hexarato a 2-ceto-glutarato-semialdeído. Por último, a 2,5 dioxovalerato desidrogenase (EC 1.2.1.26) converte o 2-ceto-glutarato-semialdeído a 2-ceto-glutarato, com o consumo de outro NAD^+.

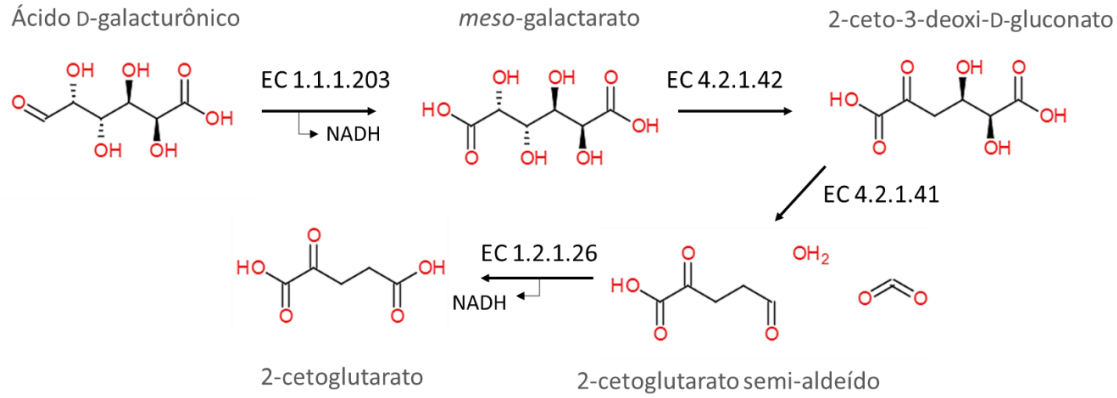

Figura 13. A via oxidativa de catabolismo de ácido D-galacturônico

De fato, já existem tentativas de construir uma cepa de *S. cerevisiae* capaz de utilizar ácido D-galacturônico. HUISJES *et al.,* (2012) deram o primeiro passo na expressão de uma via heteróloga de catabolismo do ácido D-galacturônico em *S. cerevisiae*. Eles integraram os cinco genes da via oxidativa (*uxaC*, *uxaB*, *uxaA*, *kdgK* e *kdgA*) em dois loci de *S. cerevisiae*, *URA3* e *LEU2*, sob o controle de promotores fortes e constitutivos de leveduras. A expressão de todos os genes foi observada por qPCR, mas a atividade foi confirmada somente para as duas primeiras enzimas. Além disso, não foi observado consumo de ácido D-galacturônico após 6 semanas em co-fermentação com glucose.

Devido a semelhanças evolutivas entre a levedura *S. cerevisae* e fungos filamentosos como o *A. niger* e *T. reseei*, a via redutiva é a via com mais potencial para a clonagem em *S. cerevisiae*, já que terá mais chance de as enzimas serem expressas e estarem ativas após a expressão. Desta forma, SOUFFRIAU (2012), apresentou uma estratégia baseada na expressão em *S. cerevisiae* dos quatro genes da via redutiva (de *T. reseei*) em dois plasmídeos multicópia episssomais. Três das quatro enzimas expressaram um nível satisfatório de atividade. Apesar da triagem evolutiva, por 4 semanas, em meio contendo glicerol e ácido D-galacturônico, não ocorreu consumo do ácido D-galacturônico.

As discussões acerca do transporte de ácido D-galacturônico para dentro da célula ainda são muito recentes. SOUFFRIAU *et al.* (2012) acharam evidências de que há um transporte basal de ácido D-glacturônico em *S. cerevisiae*, apesar da incapacidade desta célula de catabolizar o ácido D-galacturônico. Este transporte basal ocorreria através de um transportador do tipo “canal”, e seria aumentado em pH próximo ao pK_a do ácido D-galacturônico. Recentemente, foi descoberto um transportador específico para ácido D-galacturônico em *Neurospora crassa*, o GAT-1. Esse transportador é do tipo simporte com próton, e há evidências que outros micro-organismos, como o *A. niger*, também possuam esse transportador (BENZ *et al*., 2014).

A via redutiva produz uma molécula de piruvato e uma molécula de glicerol. O piruvato pode ser convertido diretamente para etanol por *S. cerevisiae*. Para ser capaz de produzir etanol do glicerol produzido, *S. cerevisae* possui em seu genoma dois genes cujas enzimas possibilitam a conversão do glicerol, formado pela via redutiva, a dihidroxiacetona fosfato. Essas enzimas são a glicerol quinase (EC 2.7.1.30), que ativa o glicerol a glicerol-3-fosfato, utilizando um ATP, e a glicerol-3-fosfato desidrogenase (EC 1.1.1.94), que, com o consumo de um NAD^+, oxida o glicerol-3-fosfato a diidroxiacetona fosfato. Esse composto, por sua vez, entra na glicólise, gerando um piruvato, 2 ATP e um NADH. Em condições anaeróbicas, o piruvato é convertido a acetaldeído pela piruvato descarboxilase (EC 4.1.1.1), com a liberação de um CO_2. Em seguida, o acetaldeído é reduzido a etanol, pela álcool desidrogenase (EC 1.1.1.1), com o consumo de um NADH.

A conversão da glucose a etanol em leveduras, em condições anaeróbicas, gera 2 ATP e 2 etanol, e não sobram compostos reduzidos (NADH, NADPH) ou oxidados (NAD^+, $NADP^+$), ou seja, a reação está em equilíbrio redox. O ácido D-galacturônico, por outro lado, é mais oxidado que a glucose em dois graus de oxidação. Da Tabela 2, pode-se observar que a conversão de um ácido D-galacturônico a 2 etanol gera apenas 1 ATP e exige o gasto de um NADH e um NADPH, ou seja, o balanço redox não é zero. Em razão disso, acredita-se que a produção de etanol em ácido D-galacturônico por *S. cerevisiae*, expressando a via redutiva, não será possível se o ácido D-galacturônico for a única fonte de carbono. Por outro lado, o hidrolisado de polpa cítrica possui outros açúcares que permitiriam uma co-fermentação, entre eles, glucose, frutose e galactose. A utilização desses açúcares por *S. cerevisae*, passando pela via das pentoses fosfato, aliado ao crescimento da biomassa em condições microaeróbias, formaria compostos reduzidos que poderiam compensar o desbalanço redox gerado pelo consumo de ácido D-galacturônico.

Tabela 2. Balanço redox e de ATP na conversão do ácido D-galacturônico a etanol em um mutante hipotético de S. cerevisiae, passando pela via redutiva de catabolismo do ácido D-galacturônico

Reagentes	**Produtos**	ATP	NADH	NADPH
ác. D-galacturônico	ác. L-galactônico		-1	
ác. L-galactônico	2-ceto-3-deoxi-L-galactônico			
2-ceto-3-deoxi-L-galactônico	gliceraldeído/piruvato			
gliceraldeído	glicerol			-1
glicerol	glicerol 3-P	-1		
glicerol 3-P	Dihidroxiacetona fosfato		+1	
Dihidroxiacetona fosfato	piruvato	+2	+1	
2 piruvato	2 acetaldeído			
2 acetaldeído	2 etanol		-2	
ác. D-gal.	**2 etanol**	**+1**	**-1**	**-1**

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Capítulo 2

Manuscrito artigo 1

Pectinase activity determination: An early deceleration in the release of reducing sugars throws a spanner in the works!

Short title: Questioning single-time-point pectinase assays

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ABSTRACT

Recently, it has been suggested that pectinases could be used to hydrolyze pectin in biorefineries based on pectin-rich agro-industrial wastes. However, for this to be viable, the cost of their production would need to be lowered significantly. In fact, over the last few decades, there have been many attempts to improve pectinase production by existing strains or to screen for new strains from environmental isolates. In these studies, it is necessary to measure pectinase activities. Many researchers use single-time-point assays that involve incubation of pectinolytic extracts with pectic substrates for a fixed time, followed by determination of the liberated reducing sugars. However, different researchers use quite different conditions for this assay. Furthermore, no attention has been given to the reaction profile during the assay. In the current work, we show, for the first time, that a significant deceleration in the rate of liberation of reducing sugars occurs over the first ten minutes of the reaction. As a consequence, the incubation time used in a single-time-point assay has a large effect on the value obtained for the activity. In fact, we demonstrate that, depending on the particular combination of incubation time, pectin concentration and reaction temperature, the same extract could be reported to have activities that differ by an order of magnitude. In addition, we show that the relative activities obtained with polygalacturonic acid do not correlate with those obtained with pectin. We conclude that it is currently impossible to make meaningful comparisons between pectinase activities reported in the literature by workers who have used different assay conditions. Therefore there is an urgent need for the development of a standardized assay for evaluating the saccharification potential of pectinase complexes.

Introduction

The term “pectinases” refers to a group of enzymes that act together to solubilize, de-esterify and depolymerize the complex structure of native pectin [1]. They have varied applications in the food and beverage industries, including the extraction and clarification of juices, the improvement of the organoleptic properties of wine, the increasing of the efficiency of extraction of vegetable oils and the acceleration of coffee and tea fermentations [2], [3]. Pectinases are also used as additives in animal feeds, mainly for poultry and ruminants, to improve digestibility and nutritional value [4]. Pectinases could also be used for the saccharification of pectin-rich agricultural residues, with the resulting sugars being used for the production of ethanol or platform chemicals [5]−[7]. In this manner, they would play an important role in the recently proposed citrus waste biorefineries [8], [9].

The saccharification of pectin involves a number of enzymes working in conjunction (Table 3). In the current paper, we use the term “pectinolytic activity” to denote the liberation of reducing sugars from pectin by this mixture of enzymes, which we refer to as a “pectinase complex”. The production of pectinase complexes by submerged-liquid fermentation and solid-state fermentation of various microorganisms has been studied over the last two decades or so, with many researchers having isolated new strains and undertaken medium optimization and strain improvement programs [12],[13]. In these programs, it is necessary to assay pectinolytic activity. When the interest is to produce pectinases for the food industry, it is common to determine pectinolytic activity based on measurements of the reduction in viscosity of a pectin solution [13]. Specific assays based on ruthenium red have also been developed for endopolygalacturoanases [14], [15]. However, the progress towards complete saccharification is best characterized by measuring the liberation of reducing sugars from pectin. The most frequently used method for measuring pectinase activity involves a “single-time-point” assay that requires two steps. The first step is a hydrolysis step, in which the pectinase-containing extract is incubated for a fixed time under selected conditions of pH, temperature and initial pectin concentration. In the second step, the reducing sugars liberated in the first step are quantified, either by the DNS method [16] or by the Somogyi-Nelson method [17], [18]. The advantage of this assay is that it is simple, uses relatively cheap reagents and can be used to process a relatively large number of samples simultaneously. It is performed manually in most laboratories, although a fully automated assay would be possible [19]. Activities are expressed

in terms of "μmol of D-galacturonic acid equivalents produced per minute" and reported "per mL of fermentation broth", in the case of submerged-liquid fermentation or "per gram of dry solids", in the case of solid-state fermentation (SSF).

Table 3. Enzymes that contribute to the degradation of pectin

Name	**EC number**	**Mechanism**
Pectin lyase	4.2.2.10	Eliminative cleavage of (1→4)-α-D-galacturonan methyl ester to give oligosaccharides with 4-deoxy-6-*O*-methyl-α-D-galact-4-enuronosyl groups at their non-reducing ends
Pectate lyase	4.2.2.2	Eliminative cleavage of (1→4)-α-D-galacturonan to give oligosaccharides with 4-deoxy-α-D-galact-4-enuronosyl groups at their non-reducing ends
Pectate disaccharide lyase	4.2.2.9	Eliminative cleavage of 4-(4-deoxy-α-D-galact-4-enuronosyl)-D-galacturonate from the reducing end of pectate (i.e. de-esterified pectin)
Pectate trisaccharide-lyase	4.2.2.23	Eliminative cleavage of unsaturated trigalacturonate as the major product from the reducing ends of polygalacturonic acid/pectate
Rhamnogalacturonanendolyase	4.2.2.23	Endotype eliminative cleavage of L-α-rhamnopyranosyl-(1→4)-α-D-galactopyranosyluronic acid bonds in rhamnogalacturonan I domains of hairy regions of pectin
Endo-polygalacturonases	3.2.1.15	Random hydrolysis of (1→4)-α-D-galactosiduronic linkages in pectate and other galacturonans
Exo-polygalacturonases	3.2.1.67	Hydrolysis of D-galacturonic acid residues from the reducing ends of polygalacturonate chains
Pectin methyl esterases	3.1.1.11	Demethoxylation of pectin, forming pectate
Pectin acetyl esterases	3.1.1.6	Deacetylation of pectin, forming pectate
Exo-poly-α-galacturonosidase	3.2.1.82	Hydrolysis of pectic acid from the non-reducing end, releasing digalacturonate
Rhamnogalacturonan hydrolase	3.2.1.171	Endohydrolysis of α-D-GalA-(1→2)-α-L-rha-glycosidic bonds in the rhamnogalacturonan I backbone, releasing oligosaccharides with β-D-GalA at the reducing end.
α-L-rhamnosidase	3.2.1.40	Hydrolysis of the glycosidic bonds between rhamnose and galacturonic acid residues
Arabinan endo-1,5-α-L-arabinanase	3.2.1.99	Endohydrolysis of (1→5)-α-arabinofuranosidic linkages in (1→5)-arabinans
Arabinogalactanendo-β-1,4-galactanase	3.2.1.89	Hydrolysis of (1→4)-β-D-galactosidic linkages in type I arabinogalactans.
α-L-arabinofuranidases	3.2.1.55	Hydrolysis of terminal non-reducing α-L-arabinofuranoside residues in α-L-arabinosides.

*Source: [10,11]

In order to evaluate the success of screening and strain optimization programs, it is necessary to compare the results obtained for pectinolytic activity with those reported in the literature. However, different authors carry out the hydrolysis step quite differently, with either pectin or polygalacturonic acid being used as the substrate and with diverse values being used for the pH, temperature, reaction time and substrate concentration (Table 4). These different assay conditions have consequences for comparing results that have not yet been explored. Further,

although some efforts have been made to measure initial velocities in the characterization of the kinetics of individual enzymes [37], the exact shape of the early reaction profile during assays for the liberation of reducing sugars by pectinase complexes is not clear: the profiles that have been obtained involve measurements that are rather widely spaced in time and initial rates are either not characterized or the profiles themselves not reported [38], [39]. We address these two issues in the current paper, firstly, by obtaining detailed profiles for the liberation of reducing sugars in reactions carried out under different conditions of temperature and initial pectin concentration and, secondly, by comparing reaction profiles for two commercial preparations and two crude extracts, using citric pectin and polygalacturonic acid. We identify, for the first time, the existence of a significant deceleration over the first ten minutes of the reaction and discuss the implication of this early deceleration, and other results, for the assaying of pectinolytic activities.

Materials and methods

Strains

Aspergillus niger CH4 is maintained in the culture collection of the Biomedical Research Institute of the Universidad Nacional Autónoma de México (Mexico City, Mexico) and was kindly provided by Prof. Dr. Jesus Cordova and Prof. Dr. Gustavo Viniegra-Gonzalez. *Aspergillus oryzae* CPQBA 394-12 DRM 01 was isolated from a passion fruit peel and is maintained in the culture collection of the University of Campinas (Campinas, Brazil).

Substrates for solid-state fermentations

Wheat bran was purchased from the municipal market of Curitiba, located in the state of Paraná, Brazil, and was used as obtained. Sugarcane bagasse was obtained from Destilarias Melhoramentos S/A (Jussara, Brazil) and was sieved to recover particles between 1.7 and 1 mm. Citrus pulp was obtained from the agro-industrial cooperative Corol (Rolândia, Brazil) and was dried to 13% (w/w) moisture (wet basis).

Table 4. Hydrolysis conditions used in assays for pectinase activities reported in the literature

Microorganism (cultivation mode)*	**Pectic substrate & concentration (% m/v)#**	**T (°C)**	**t (min)**	**pH**	**Activity (SSF: U g^{-1}; SLF: U mL^{-1})**	**Reference**
Thermomucor indicae-seudaticae (SSF)	1% pectin	60	10	5.5	108	[20]
Penicillium viridicatum (SSF)	0.8% pectin	50	10	5.5	71	[21]
Thermoascus aurantiacus (SSF)	0.8% pectin	55	10	5.0	43	[22]
Fusarium moniliforme (SSF)	0.5% pectin	40	30	4.5	43	[23]
Aspergillus niger (SSF)	0.45% pectin	45	30	4.5	25	[24]
Aspergillus awamori (SSF)	0.33% pectin	45	10	5.0	9	[25]
Streptomyces sp. (SLF)	1% pectin	60	-	3	162	[26]
Penicillium occitanis (SLF)	0.45% pectin	50	60	4.8	64	[27]
Aspergillus niger (SLF)	0.5% pectin	-	15	4.5	34	[28]
Aspergillus flavipes (SLF)	1% pectin	45	20	5	20	[29]
Aspergillus niger (SLF)	0.2% pectin	30	-	5.3	1.3	[30]
Trichoderma viridae (SlSF)	0.2% PGA	30	-	5	10	[31]
Aspergillus sojae (SSF)	0.2% PGA	26	-	6.6	30	[32]
Bacillus subtilis (SSF)	0.24% PGA	65	10	9.5	6592	[33]
Aspergillus niger (SSF)	-	40	-	3.5	135	[34]
Aspergillus sp. (SSF)	0.17% PGA	-	10	5.3	390	[35]
Penicillium grisereoserum (SLF)	0.75% PGA	40	20	4.8	3490	[36]

*SSF: solid-state fermentation; SLF: submerged fermentation; SlSF: slurry-state fermentation
#PGA: polygalacturonic acid

Preparation of crude extracts from solid-state fermentations

The medium contained 1.5 g dry sugarcane bagasse and 3.5 g of either dry wheat bran (for *A. niger* CH4) or dry citrus pulp (for *A. oryzae* CPQBA 394-12 DRM 01). The mixture was placed in a 250-mL Erlenmeyer flask and autoclaved (121°C, 15 min). Saline solution (containing, in g/L, K_2HPO_4 3, $(NH_4)_2SO_4$ 13, $MgSO_4.7H_2O$ 5, KCl 10, $FeSO_4.7H_2O$ 0.09) was autoclaved (121°C, 15 min) and added to each flask to obtain a final moisture content of 70% (w/w, wet basis). The solid media were inoculated with 10^7 spores per gram of dry substrate and incubated

at 30°C for 24 h. The fermented solids were then lyophilized and stored at 4°C. For the extraction, 100 mL of acetate buffer (0.2 M, pH 4.5) was added per gram of lyophilized fermented solids and the mixture was incubated in an orbital shaker at 30°C for 30 min at 180 rpm. The crude extracts were vacuum filtered in Whatman n°1 filter paper. The filtrate was stored at 4°C.

Commercial enzymes and pectic substrates

The commercial enzyme preparations used were Pectinex® Ultra SPL (Novozymes, Denmark), from *Aspergillus aculeatus*, and Pectinase P4716 (Sigma-Aldrich, USA), from *Aspergillus niger*. These preparations were diluted 2500-fold in acetate buffer (0.2 M, pH 4.5) before adding to the substrate mixture. Citric pectin (containing at least 75% D-galacturonic acid) and polygalacturonic acid (Sigma-Aldrich, USA) were solubilized in acetate buffer (0.2 M, pH 4.5).

Determination of the profiles for the release of reducing sugars

The studies of the effect of temperature and pectin concentration on the reaction profile were undertaken using the crude extract of *A. niger* CH4. For this, multiple identical tubes were prepared, each containing 0.25 mL of crude extract and 0.25 mL of citric pectin in acetate buffer (0.2 M, pH 4.5). The stated pectin concentration represents the value for the mixture at zero time. The samples were incubated at the stated temperature. At 30 s intervals, 0.5 mL of DNS was added to the mixture to stop the reaction. All the profiles were obtained with aliquots taken from the same crude extract.

In the study of initial hydrolysis profiles undertaken with different pectinase complexes and pectic substrates, 15 mL of the appropriate substrate solution was incubated in a 100-mL jacketed reactor at 30°C. To start the reaction, 15 mL of the appropriate pectinase preparation was added, giving an initial substrate concentration in the reaction mixture of 0.5% (m/v). Samples of 0.5 mL were taken at intervals of 10 s (0-2 min), 30 s (2-15 min) or 1 min (15-37 min). Each sample was added to 0.5 mL of DNS in an ice bath to stop the reaction.

In the study of long hydrolysis times that was undertaken with different pectinase complexes and pectic substrates, 75 mL of the appropriate substrate solution was incubated in a 250-mL

Erlenmeyer flask at 30°C. To start the reaction, 75 mL of the appropriate enzyme preparation was added, giving an initial substrate concentration of 0.5% (m/v). The mixture was incubated on an orbital shaker at 200 rpm and 30°C for 72 h. Samples of 0.5 mL were taken at various times and added to 0.5 mL of DNS on ice bath to stop the reaction.

The liberation of reducing sugars was followed using the DNS method [16]. Zero time absorbances were subtracted from the readings. Reducing sugar concentrations were estimated as μmol of D-galacturonic acid equivalents using a calibration curve constructed with D-galacturonic acid (Sigma-Aldrich, ≥ 98.0% purity) concentrations from 92 to 920 μmol mL^{-1}.

Determination of corresponding single-time-point activities from reaction profiles

A fifth-order polynomial was fitted to the reaction profile obtained under each set of conditions. For all fits, the value of R^2 was ≥0.90. The fitted curve is only shown in Fig. 1; for the sake of clarity, other fitted curves are not shown, since the graphs already contain several different plots. The activity that would have been obtained in a single-time-point assay undertaken using a particular time under a particular set of reaction conditions, denominated $A(t)$ and with units of U g^{-1}, was then calculated as:

$$A(t) = \frac{C(t) \times D \times R}{t} \quad (1)$$

where $C(t)$ is the reducing sugar concentration (μmol mL^{-1}) at time t (min), obtained by substituting t into the corresponding fitted polynomial, D is the dilution factor for the extract in the assay (equal to 2) and R is the ratio of buffer to dry solids used in the extraction step (mL g^{-1}, equal to 100). Note that 1 U corresponds to the production of 1 μmol of D-galacturonic acid equivalents per min.

Stability assays

The crude extract obtained from SSF of *A. niger* CH4 was incubated for 10, 30 and 60 min at 30, 40, 50, and 55°C. The assay for residual activity was undertaken at 50°C, for 10 min, using 0.5% (w/v) pectin (final concentration in the hydrolysis solution). The residual activity was

calculated relative to the activity obtained in an assay undertaken under the same conditions but without previous incubation.

Results

Reaction profiles under different conditions

Since pectin degradation profiles that have been published in the literature typically only provide data points at intervals of several minutes, the first experiment involved the characterization of the degradation of pectin by a crude extract from *A. niger* CH4, using intervals of only 30 s. It was done at 40°C and with a pectin concentration of 0.5% (w/v). A key result, which has not been previously reported in the literature, was that the rate of liberation of reducing sugars decelerated significantly over the first 10 min (Fig. 14). From 10 to 40 min there was an almost linear increase in reducing sugar concentration.

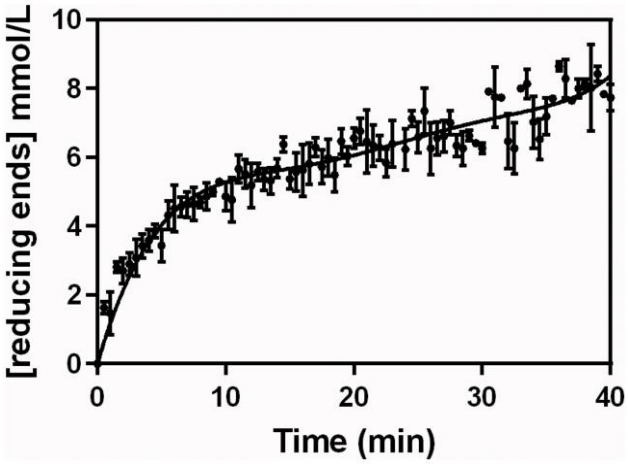

Figure 14. Detailed profile for the hydrolysis of pectin during the first 40 min of the reaction.
The hydrolysis was performed at 40°C, with an initial pectin concentration of 0.5% (w/v), using the pectinase complex produced by *Aspergillus niger* CH4 in solid-state fermentation. The reaction was undertaken in triplicate. The mean values for each time were plotted, with the error bars representing the standard error of the mean. The curve represents the best fitting fifth-order polynomial.

Reaction profiles were also followed at various different temperatures, using a pectin concentration of 0.5% (w/v). As in the previous experiment, in all conditions there was a significant deceleration over the first 10 min (Fig. 15). The effect of temperature on the liberation of reducing sugars was greatest over this 10 min period, with the amount of reducing

sugars at 40 and 50°C being slightly higher than that at 30°C. From 10 min onwards, the profiles had similar slopes.

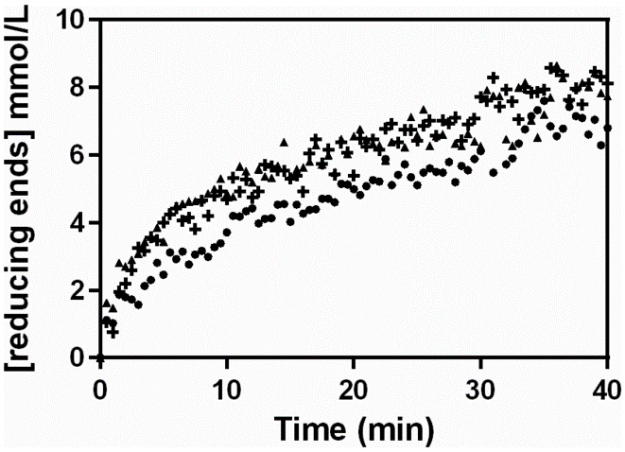

Figure 15. Reaction profiles obtained over the first 40 min at different temperatures
The hydrolysis was performed using 0.5% (m/v) pectin, with the pectinase complex produced by *Aspergillus niger* CH4 in solid-state fermentation. Symbols: (●) 30°C, (✚) 40°C and (▲) 50°C.

Reaction profiles were then followed, at 45°C, with different initial pectin concentrations (Fig. 16). The initial reaction rate was highest with 1% (w/v) pectin. However, this concentration led to a viscous solution, which made pipetting difficult, and undissolved pectin was visible suspended in the reaction medium. These problems are probably responsible for the greater dispersion of data points in the 1% (w/v) profile in Fig. 16, compared to the profiles obtained at lower initial pectin concentrations. Again, in all the conditions a significant deceleration occurred over the first 10 min.

Effect of temperature on loss of pectinolytic activity

Increasing temperatures not only increase the intrinsic rate of enzyme-catalyzed reactions, but also increase the rate of enzyme denaturation. In order to investigate the degree to which denaturation might be occurring in the profiles in Fig. 15, the extract was incubated at various temperatures (Fig. 17). The pectinase activity was stable over 60 min at 30°C and 40°C. At 50°C, the residual activity fell significantly, reaching only 25% at 60 min. At 55°C, denaturation was significantly faster, with only 6% residual activity at 10 min. These results suggest that thermal denaturation is not the cause of the early deceleration in Fig. 14, since that

reaction was carried out at 40°C, but that some denaturation probably occurred in the reactions carried out at higher temperatures in Figs 15 and 16.

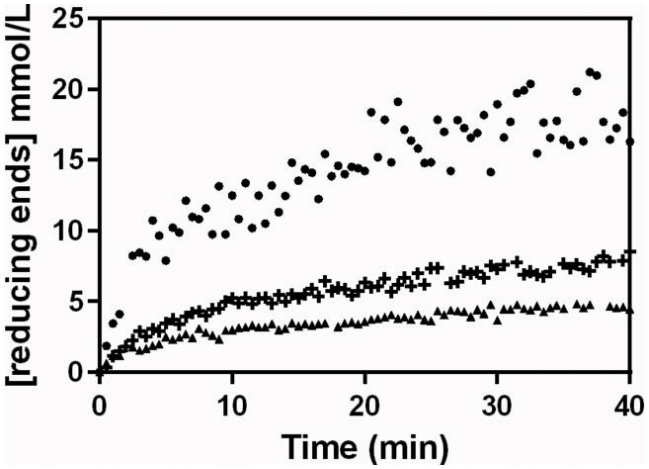

Figure 16. Reaction profiles obtained over the first 40 min with different initial pectin concentrations
The hydrolysis was performed at 45°C, with the pectinase complex produced by *Aspergillus niger* CH4 in solid-state fermentation. Symbols: Initial pectin concentrations of (▲) 0.25% m/v, (✚) 0.5% m/v and (●) 1% m/v.

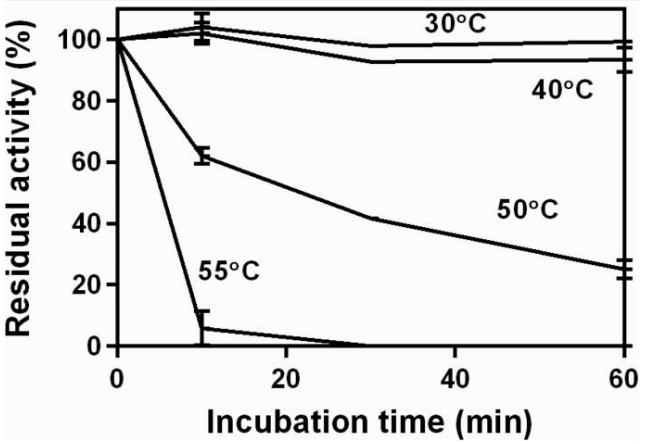

Figure 17. Residual activity curves for the extract incubated over 60 min at different temperatures
The pectinase complex produced by *Aspergillus niger* CH4 in solid-state fermentation was used. Residual activities were obtained at 50°C, in a 10-min assay, using 0.5 % pectin (w/v).

Consequences of determining pectinolytic activity using single-time-point assays

The reaction profiles obtained above were used to calculate the pectinolytic activities that would be obtained in single-time-point (STP) assays, carried out under various combinations of temperature and pectin concentration that are typically used in the literature (see Table 4). These will be referred to as "STP-activities". STP activities were calculated for each of three commonly used reaction times, 10, 15 and 30 min (Table 5). In analyzing these results, it is

important to note that all assays involve the same amount of enzyme, with the differences in STP-activity being due to the conditions and the time chosen for the reaction. The STP-activity was influenced most by the reaction time and the concentration of pectin. Importantly, the highest value of STP-activity, obtained for 1.0% (w/v) pectin, 45°C and 10 min, was 8-fold greater than the lowest value of STP-activity, obtained for 0.25% (w/v) pectin, 45°C and 30 min.

Table 5. Single-time-point pectinase activities that would be obtained for the same enzymatic extract incubated under different reaction conditions for different times

Reaction conditions		**"Single-time-point activity" (STP-activity) (U/g) at the stated reaction times***		
[pectin] (%, w/v)	**T (°C)**	**10 min**	**15 min**	**30 min**
0.25	45	28.0	22.0	14.5
0.5	30	38.1	30.5	20.2
0.5	40	50.6	38.8	23.3
0.5	45	47.6	37.3	24.3
0.5	50	46.5	36.2	24.4
1.0	45	114.4	86.4	56.1

*Calculated from the data in Figs 1 to 3 using Eq. (1).

Early profiles for different pectinase preparations acting on different pectic substrates

To evaluate how the type of pectic substrate affects the activity, profiles for the liberation of reducing sugars were obtained for both polygalacturonic acid and citric pectin. Based on the results in Figures 14 to 17, the conditions chosen for the reactions were 0.5% (m/v) pectic substrate and 30°C. Profiles were obtained with four different pectinase complexes: a crude extract from *A. niger* CH4 (maintained as a reference), a crude extract from *A. oryzae*, Pectinase P4716 and Pectinex Ultra SPL. These pectinase complexes were diluted so as to give similar profiles on polygalacturonic acid (Fig. 18A). The same dilutions were then used for the degradation of citric pectin (Fig. 18B).

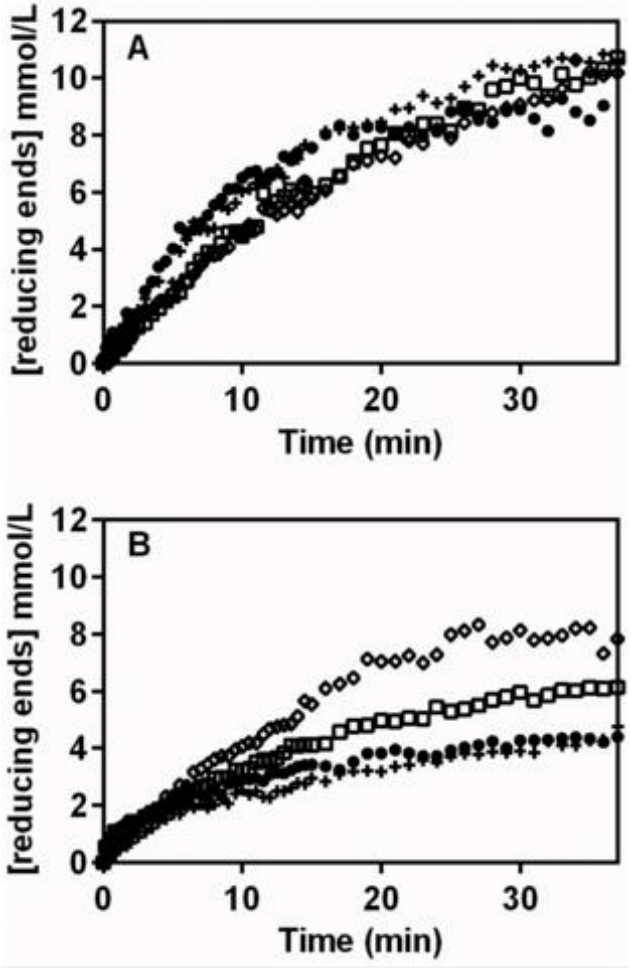

Figure 18. Short hydrolysis profiles (40 min), obtained using different pectinase complexes and either polygalacturonic acid (A) or citric pectin (B)
The hydrolysis was performed at 40°C, with an initial substrate concentration of 0.5% (w/v). The pectinase complexes used were the extracts of *Aspergillus niger* (●) and *Aspergillus oryzae* (✚), produced in solid-state fermentation, and the commercially available Pectinex Ultra SPL (Novozymes) (□) and Pectinase P4716 (Sigma-Aldrich) (◇).

Three key observations can be made. First, all profiles in Figs 18A and 18B show the early deceleration observed in Figures 14, 15 and 16. Second, comparing the same pectinase complex on different substrates, the rate of liberation of reducing sugars from citric pectin was lower than that obtained with polygalacturonic acid. Third, comparing different pectinase complexes on the same substrate, although the amounts of these complexes were chosen so as to give similar hydrolysis profiles with polygalacturonic acid, when the same amounts were added to citric pectin, the profiles obtained were quite different from one another. The most effective hydrolysis of citric pectin was obtained with Pectinase P4716.

Extended profiles for different pectinase preparations acting on different pectic substrates

The same amounts of pectinase complex that were used for the hydrolysis of polygalacturonic acid in Fig. 5A were added to 0.5% (m/v) of the two pectic substrates and the reactions were followed for 72 h at 30°C. The results are plotted for 24 h since in all cases there was no further liberation of reducing sugars after this time (Fig. 19A and B).

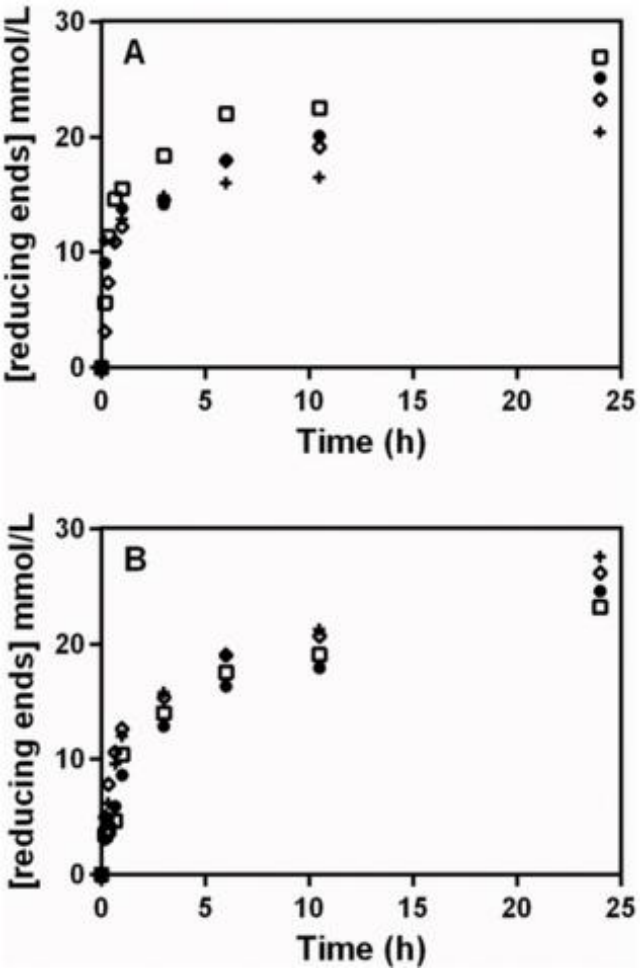

Figure 19. Extended hydrolysis profiles (24 h), obtained using different pectinase complexes and either polygalacturonic acid (A) or citric pectin (B)
The hydrolysis was performed, at 40°C, with an initial substrate concentration of 0.5% (w/v). The pectinase complexes used were the extracts of *Aspergillus niger* (●) and *Aspergillus oryzae* (✚), produced in solid-state fermentation, and the commercially available Pectinex Ultra SPL (Novozymes) (□) and Pectinase P4716 (Sigma-Aldrich) (◇).

Two key observations can be made from Fig 19. First, there is a continued deceleration of the reaction up to 6 h, but this deceleration is less pronounced than that which occurred over the first 10 min in Figs 1, 2, 3 and 5. This is followed by a slow, essentially constant rate of reducing

sugar release from 6 to 24 h. Second, although Pectinase P4716 liberated significantly more reducing sugars from citric pectin than did the other complexes over the first 40 min of reaction (Fig. 18B), this superior efficiency was not maintained. In fact, at 24 h, slightly more reducing sugars had been liberated by the extract from *A. oryzae* than by Pectinase P4716.

Discussion

Our work makes two main contributions. First, we have demonstrated that, during the hydrolysis of polygalacturonic acid and citric pectin by pectinase complexes from various sources, there is a significant deceleration in the rate of release of reducing sugars over the first ten minutes, followed by a longer, slower, deceleration over several hours. Second, we have identified several important issues with respect to the use of the single-time-point assays that are commonly used for determining pectinase activities. These contributions are discussed below.

Implications of the early deceleration

Although the release of reducing sugars from pectic substrates by pectinase complexes has already been studied [38] − [40], in these studies the reaction profiles were obtained by taking samples at intervals of up to ten minutes or more. By removing samples at 30-s intervals, we have, for the first time, demonstrated that there is a significant deceleration in the rate of release of reducing sugars over the first ten minutes of reaction.

This early deceleration has important consequences for the determination of pectinase activities by measuring the liberation of reducing sugars in STP-assays. As Table 5 shows, for the same pectinase preparation and for any given combination of reaction conditions, the activity that would be determined in an STP-assay decreases significantly as the incubation time is increased. This occurs because increases in reaction time above 10 min are associated with proportionally small increases in the reducing sugar level, so the result given by Eq. (1) becomes increasingly smaller.

Interestingly, the early deceleration is not apparent in the profiles that Baciu and Jördening [41] presented for the release of D-galacturonic acid from pectin by a different Pectinex product (Pectinex® 100 L, Novozymes). Their profiles cover the first 300 min and show a gradual deceleration over this time. Our experiments differ from theirs in an important aspect: we measured the liberation of reducing sugars and therefore detected not only D-galacturonic acid in itself, but also oligomers produced by endo-acting enzymes. Baciu and Jördening attributed the gradual deceleration to product inhibition by D-galacturonic acid. This is likely to be the cause of the slow deceleration that occurred over longer hydrolysis times in the current work (see Fig. 6). However, further research is required to determine whether the early deceleration in the liberation of reducing sugars is also caused by product inhibition.

Other issues related to the use of single-time-point assays for determining pectinase activities

As Table 5 shows, in STP-assays, the same pectinase preparation could be reported as having a pectinolytic activity ranging over almost an order of magnitude, depending on the particular combination of hydrolysis conditions that is chosen. Since previous researchers have used not only different reaction times in carrying out STP-assays for pectinase activity, but also different temperatures and pectin concentrations (Table 4), it is impossible to draw meaningful conclusions about which result from the literature actually represents the best pectinase production. A lower amount of pectinases, assayed under favorable conditions, could lead to an activity higher than that obtained for a higher amount of pectinases, assayed under unfavorable conditions.

This raises an important question for researchers interested in using pectin-rich agro-industrial wastes in biorefineries. One of the processing steps in such biorefineries is likely to be the saccharification of pectin, for which it is desirable to identify good pectinase-producing microorganisms: Is it possible to establish a standard assay for screening pectinase activities that will not only reflect the saccharifying potential of a pectinase preparation but can also be used to enable direct comparisons with the results of other research groups? It is useful to compare the current situation with respect to pectinase activity determination with the situation of cellulase activity determination in the 1980's. At that time there was much interest in the production of ethanol from cellulosic biomass, but different investigators used different assays and different conditions to determine cellulase activities. Then, in 1987, Ghose [42] proposed

standardized methods for the determination of cellulase activity. Most workers in the field of cellulases now use these methods, allowing meaningful comparisons to be made within the cellulase literature.

Some authors have used polygalacturonic acid as the substrate in assays for pectinolytic activity [36], [40], [43], [44]. On the basis of our results, we recommend that in any assay that aims to evaluate the potential for pectinase preparations to saccharify pectin, the substrate used in the assay should be pectin and not polygalacturonic acid: the activities obtained with polygalacturonic acid are higher than those obtained with pectin, so the use of polygalacturonic acid in assays would lead to an overestimation of activities. Furthermore, the pectinase complex that hydrolyzes polygalacturonic acid most efficiently might not be the pectinase complex that hydrolyzes pectin most efficiently.

It would be a relatively simple matter to select appropriate values for pectin concentration, temperature and pH for a standard pectinase assay based on the liberation of reducing sugars. The pectin concentration should be as high as possible without causing problems with viscosity (e.g. 0.5% w/v). The temperature should not cause denaturation (e.g. a value in the range of 30 to 40°C). Table 3 shows that many authors are performing activity assays at temperatures of around 50°C or more, which are likely to lead to significant denaturation of the pectinases during the assay. Although these temperatures lead to high activity values over the short period of the assay, they are unlikely to be used in saccharification processes of up to 24 h or more, unless the enzymes in the preparation are thermostable. The effect of pH on pectinase activity was not studied in the current work because it has already been well studied [38], [39], [45], [46]. Most pectinases have a pH optimum around 4.5. However, some pectinases, principally those produced by species of *Bacillus*, have a pH optimum between 8 and 10.5 [47], [48].

On the other hand, it is not clear what would be an appropriate reaction time for a standard pectinase assay. Several possibilities might be considered. For example, initial velocities could be determined. However, due to the non-linearity of the reaction profile, it would be necessary to obtain various data points over the first minutes of the reaction in order to be able to estimate the initial velocity with an acceptable precision. In any case, since the greater part of the saccharification process occurs over many hours, after a significant initial deceleration, initial velocities are unlikely to reflect the long-term saccharification potential.

Of course, assays could be carried out over 24 h. In this case, it would be unwise to use a single time point at 24 h, since the reaction might reach completion before this time. It would be advisable, therefore, to have various measurements during this time. However, such a long reaction time would be inconvenient in screening programs, as it would greatly reduce throughput. Researchers are likely to prefer to use such long reaction times in monitoring process development, not in initial screening programs.

One possibility might be to use a "double-time-point" assay. A first reading could be taken after the initial deceleration, at 20 min or so. A second reading could then be taken later, maybe at 40 min or even at 60 min. The activity could then be expressed on the basis of the difference between these two readings. This would not be as convenient as an STP-assay, but would have the advantage of not including the period during which the early deceleration occurs. However, it remains to be proven whether activities obtained in this manner would reflect the long-term saccharification potential.

Conclusions

We have shown, for the first time, that there is a significant early deceleration in the liberation of reducing sugars from pectic substrates by pectinase complexes. On the basis of this phenomenon, as well as results regarding the effects of temperature and substrate concentration on the reaction profile, we have shown that it is not possible to make meaningful comparisons between pectinase activities reported by different authors who have used different assay conditions, substrates and reaction times in single-time-point assays. There is an urgent need for the establishment of a standardized assay procedure for evaluating the saccharification potential of pectinase complexes.

Acknowledgements

Research scholarships were granted to A. Biz by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), a Brazilian government agency for the development of personnel in higher education, and to F.C. Farias, F.A. Motter, D.H. Paula, N. Krieger and D.A. Mitchell by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), a Brazilian

government agency for the advancement of science. The project was funded by CNPq in the form of a research grant and by both CNPq and the Academy of Finland in the form of a cooperation project.

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Capítulo 3

Manuscrito artigo 2

Production of pectinases by solid-state fermentation on a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor

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ABSTRACT

Pectinases can be used in citrus waste biorefineries to hydrolyze the pectin in citrus pulp to produce D-galacturonic acid, a potential platform chemical. Solid-state fermentation has the potential to produce low-cost pectinases for such biorefineries, but it is difficult to control the process at large scales. In our work, we cultivated *Aspergillus oryzae* in a pilot-scale packed-bed bioreactor, on 15 kg of a substrate containing 51.6 % citrus pulp and 48.4 % sugarcane bagasse (w/w, dry basis). The sugarcane bagasse gave a high bed porosity and ensured a stable bed structure, avoiding problems of bed shrinkage and the formation of compact agglomerates within the bed. As a result, bed temperatures were controlled to within 1°C of the inlet air temperature and pectinase yields of 33 to 41 U g^{-1} were obtained across the bed. When the fermented solids were dried and added directly to a pectin solution, they gave a profile for the release of D-galacturonic acid similar to that obtained with a commercial pectinase. These results show the potential for using solid-state fermentation to produce pectinases in a citrus waste biorefinery, with subsequent direct addition of the fermented solids to produce D-galacturonic acid from the pectin contained in the citrus pulp.

Keywords: pectinases, *Aspergillus oryzae*, solid-state fermentation, packed-bed bioreactor, scale-up, enzyme production.

Introduction

Citrus pulp is produced in considerable amounts in Brazil and the USA: in the 2014/2015 harvest, these two countries produced about 1.34 million metric tons of dry citrus pulp (calculated from [1]). Currently, citrus pulp is either dumped, or given away or sold with a very small margin to be used as a complement for cattle feed [2]. However, citrus waste biorefineries could be established to produce pectin [3], limonene [3-5], ethanol [3-5] and D-galacturonic acid as a platform chemical [6,7].

A key step in citrus wastes biorefineries is the hydrolysis of pectin, liberating D-galacturonic acid. It is necessary to use the enzymatic route for this hydrolysis, since chemical hydrolysis leads to significant degradation of the liberated sugars [8]. However, in order to maximize the

economic performance of the biorefinery, it will be essential to minimize the costs of producing the pectinase preparation that is used for pectin hydrolysis.

One strategy for reducing the costs of producing pectinases is to use solid-state fermentation (SSF). However, although there have been numerous studies of pectinase production by SSF at laboratory scale [9], few attempts have been made to scale up this process. This is not surprising: since general strategies for scale-up of SSF processes are not yet available, processes must be scaled up on a case-by-case basis. In fact, studies of pectinase production involving more than 10 kg of dry substrate have only been carried out by Huerta et al. [10], He and Chen [11] and Pitol et al. [12]. The last of these investigations was undertaken by our group and represents the largest scale for production of pectinases by SSF that has been used to date. In that work, we grew *Aspergillus niger* in a pilot-scale packed-bed bioreactor, using 30 kg (dry matter) of a substrate consisting of 90% wheat bran and 10% sugarcane bagasse. However, these fermentations suffered from problems with the formation of agglomerates of substrate particles and with shrinkage of the bed. These problems led to overheating of parts of the bed and a consequent lack of uniformity of pectinase levels within the bed at the end of the fermentation. The aim of the current work was to avoid these problems by producing pectinases using a substrate with better mechanical properties, namely a mixture of 51.6 % citrus pulp and 48.4 % sugarcane bagasse. The organism used was *Aspergillus oryzae*, which produces high pectinase yields on this substrate mixture [13].

Materials and methods

Raw materials

Sugarcane bagasse and citrus pulp were kindly donated by Usina de Álcool Melhoramentos (Jussara, Brazil) and Corol Cooperativa Agroindustrial (Rolândia, Brazil), respectively. These products were sun and oven dried, respectively, and then used without further preparation.

Fungal strain

The strain used was *Aspergillus oryzae* CPQBA 394-12 DRM 01, originally isolated from decomposing passion fruit peels and identified by CPQBA–UNICAMP (Campinas, Brazil).

Solid-state fermentation in the column bioreactor

Throughout this work, moisture contents were determined in an infrared balance (Gehaka IV 2000, São Paulo, Brazil) and are reported on a wet basis. The solid medium contained 48.4% of sugarcane bagasse and 51.6% (w/w, dry matter) citrus pulp by mass. The solid medium was autoclaved (121°C, 15 min), in various beakers, with each beaker containing enough material to load one column, namely 9.16 g of substrate (corresponding to 8 g dry matter). $(NH_4)_2SO_4$ solution was autoclaved (121°C, 15 min) and added to each beaker to obtain a moisture content of 78.4% (w/w) and a $(NH_4)_2SO_4$ concentration of 3.48% (w/w, based on total dry substrate). The solid medium in each beaker was inoculated with a spore suspension to obtain a concentration of 4×10^7 spores per gram of dry substrate and mixed thoroughly. Initially, SSF was performed in glass columns (internal diameter of 4 cm and height of 21 cm). Each column was packed with the inoculated solid from one beaker and placed in a water bath set at 30ºC. A continuous stream of water-saturated air was injected into the bottom of each column at a rate of 100 $cm^3\ min^{-1}$. A column was sacrificed at each sampling time. The CO_2 production and O_2 consumption were continuously monitored using infrared and electrochemical sensors, respectively (PASPORT Carbon Dioxide Gas Sensor PS-2110 and PASPORT Oxygen Gas Sensor PS-2126A, PASCO scientific, California, USA). The rate of metabolic heat production was calculated based on a factor of 520 kJ metabolic heat generated per mol O_2 consumed [14]. The RQ (respiratory quotient) was calculated by dividing the CO_2 evolution rate by the O_2 uptake rate [15].

Preparation of the inoculum for the pilot-scale bioreactor

Twenty 500-mL Erlenmeyer flasks, each containing 20 g of a mixture of 48.4% sugarcane bagasse and 51.6% citrus pulp (w/w), were autoclaved (121°C, 15 min). $(NH_4)_2SO_4$ solution was autoclaved (121°C, 15 min) and added to each flask to obtain a moisture content of 60% (w/w) and a final $(NH_4)_2SO_4$ concentration of 3.48% (w/w, based on total dry substrate). A

spore suspension was added to give 4×10^7 spores per gram of dry substrate and the flasks were incubated at 30°C until sporulation (approximately 5 days). The spores were resuspended in sterile distilled water and then filtered through sterile gauze to remove residual substrate. The spore concentration in the suspension was determined using a Neubauer chamber. This process gave 6 L of spore suspension with 1×10^{11} spores per liter.

Solid-state fermentation in the pilot-scale bioreactor

Duplicate pilot-scale fermentations were undertaken in the same pilot-scale bioreactor as that used by Pitol et al. [12] and is described in Figure 20. The bed within this bioreactor has a horizontal-cross section of 60 cm by 70 cm and can have a height up to 50 cm (Fig. 20B). A 32°C-reservoir was used to feed the humidification column. The cool water reservoir was not used in the fermentations. The air flow rate was maintained at 150 m^3 h^{-1}, resulting in a superficial air velocity of 0.1 m s^{-1}. The CO_2 evolution was continuously monitored using the PASCO PS-2110 infrared sensor (Fig. 20A).

The substrate, 15 kg (dry mass), contained 7.26 kg of sugarcane bagasse and 7.74 kg of citrus pulp, 522 g $(NH_4)_2SO_4$ and 49.45 L of water. This gave proportions of 51.6% citrus pulp, 48.4% sugarcane bagasse, an $(NH_4)_2SO_4$ content of 3.48% (w/w, based on total dry substrate) and a moisture content of 78.4% (w/w). This solid medium, the water and the $(NH_4)_2SO_4$ solution were autoclaved at 121°C for 2 h. After cooling, the solid medium, the water, the $(NH_4)_2SO_4$ solution and sufficient spore suspension needed to give 4×10^{10} spores per kg of dry substrate were added to the bioreactor. The bioreactor was then rotated for 2 min at 5 rpm to homogenize the bed. After this period, the bed surface was leveled using a rake, the bioreactor was closed and the air supply was connected. During the fermentation, samples for the determination of pectinase activity were collected only from the top of the bed, in order to avoid the creation of preferential paths for air flow. At the end of the fermentation, samples were removed from five different horizontal positions (C1, C2, C3, C4 and C5) near the bottom (5 cm), in the middle (20 cm) and at the top (40 cm) of the bed to verify the uniformity of pectinase activities and moisture contents in the bed (Fig 20C).

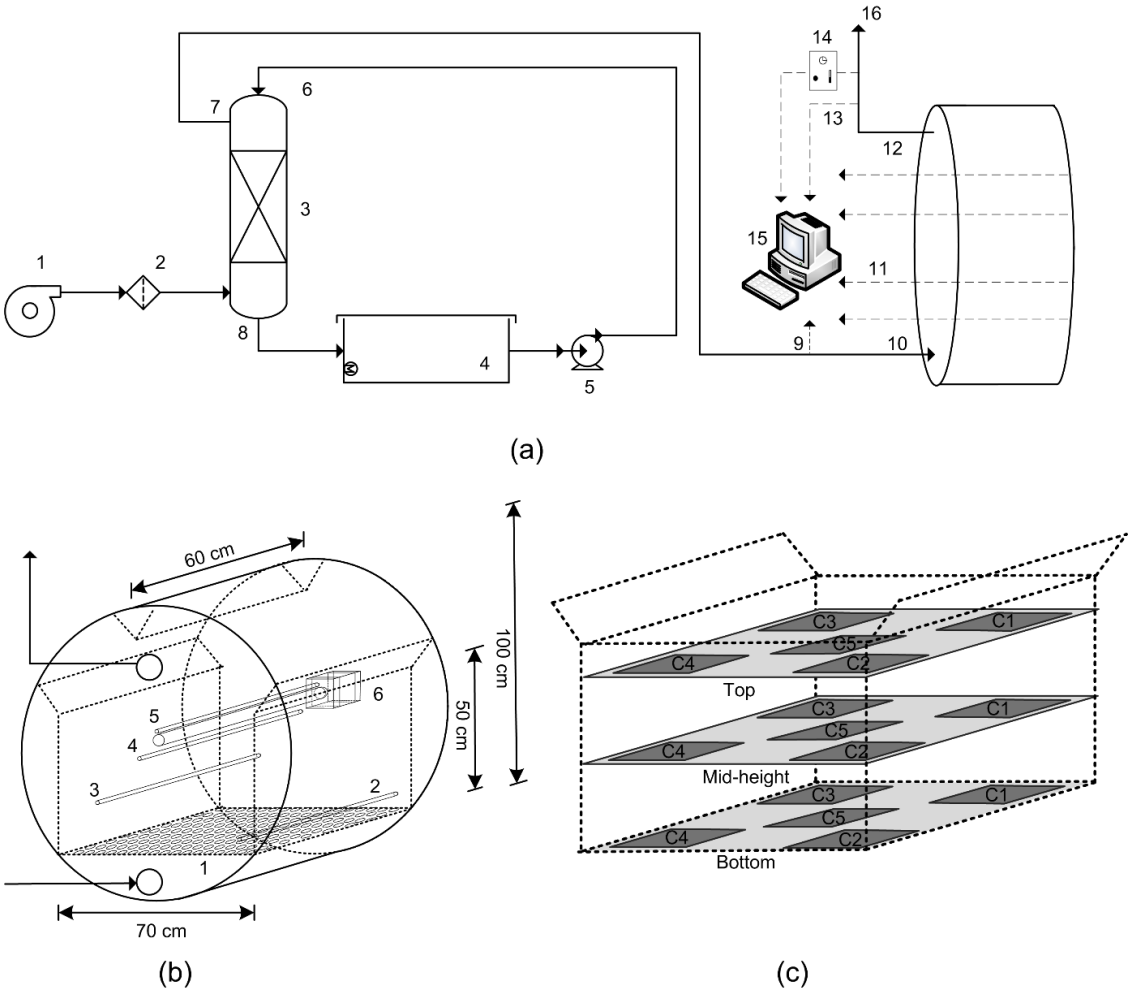

Figure 20. The pilot-scale bioreactor
(a) Overview of the bioreactor and the air preparation system. Key: (1) air blower; (2) air filter; (3) humidification tower; (4) warm water reservoir; (5) warm water pump; (6) water entry into the humidification tower; (7) supply of warm saturated air to bioreactor; (8) return of excess water back to the corresponding reservoir; (9) measurement of inlet air temperature; (10) air inlet (11) measurements of bed temperature; (12) air outlet; (13) measurement of outlet air temperature; (14) oxygen sensor; (15) data acquisition equipment; (16) to gas washer. (b) Design details of the pilot-scale packed-bed bioreactor; Key: (1) 1-mm aperture wire mesh supported on a perforated base plate; (2) thermocouple at 5 cm height; (3) thermocouple at 18 cm height; (4) thermocouple at 33 cm height; (5) thermocouple at 46 cm height; (6) electric motor with variable frequency; (c) locations where the samples were removed at the end of the fermentation for mapping the bed. This figure was originally provided by Pitol et. al [12], and is reproduced here with authorization of Elsevier.

Determination of pectinase activities

Twenty milliliters of acetate buffer (0.2 M, pH 4.5) was added to samples of 1 g of dry substrate. The extraction was carried out at 160 rpm and 30°C for 30 min. The crude extracts were vacuum-filtered through gauze. For the pectinase assays, 0.25 ml of the properly diluted crude extract was added to 0.25 mL of 1% (w/v) citric pectin (Sigma, 75% methylation) in acetate buffer (0.2 M, pH 4.5) and incubated for 20 min at 30ºC. A standard curve of D-galacturonic

acid (Sigma-Aldrich, ≥ 98.0% purity) was used, and the release of reducing sugars was analyzed using the DNS method [16]. One unit (U) of pectinase activity corresponds to the release of 1 μmol of reducing ends per minute. Activities are expressed on the basis of the mass of dry substrate (i.e. U g^{-1}). Results are given as the means of triplicate determinations with the error being reported as the standard error of the mean.

Pectin hydrolysis

After the second pilot-scale fermentation, approximately 1 kg of fermented solid was collected randomly from several positions of the bed and lyophilized for 24 h, at -45°C and 0.1 mbar (Jouan LP3 Lyophilizer, Allerød, Denmark). An extract was prepared using 5 g of this lyophilized solid and 100 mL of 200 mM acetate buffer pH 4.5. In addition, the commercial pectinase Pectinex® (Novozymes, Denmark) was diluted 500-fold in 200 mM acetate buffer pH 4.5. Three hydrolysis reactions were performed: (i) 0.5 g of lyophilized fermented solid added directly to 100 mL of pectin solution; (ii) 10 mL of extract from the lyophilized fermented solid added to 90 mL of pectin solution; (iii) 10 mL of the diluted Pectinex® added to 90 mL of pectin solution. All pectin solutions were prepared so as to give concentrations of 2% (w/v) at the start of the reaction. The hydrolyses were performed in triplicate in a shaker at 160 rpm, 30°C for 48 h. Samples were taken at intervals, boiled for 5 min to inactivate the enzymes, and then centrifuged to remove solids. The reducing sugars in the supernatants were analyzed by the DNS method [16] and the D-galacturonic acid contents were determined by HPLC using a 1260 Infinity Bio-Inert Quaternary LC System (Agilent, Santa Clara, California, USA) fitted with a Cation-H pre-column linked to an ion exchange column Hi Plex H (Agilent, Santa Clara, California, USA) maintained at 65°C. The eluent was 5 mmol L^{-1} H_2SO_4 at a flow rate of 0.55 mL min^{-1}. Peaks were detected using a differential refractometer at 40°C.

Results

Preliminary studies

At the start of this work, we were producing pectinases from *Aspergillus oryzae* grown on a mixture of 30% sugarcane bagasse and 70% citrus pulp, with an initial moisture content of 70%

and supplemented with a complex salt solution. With this medium and initial conditions, pectinase activities of 15 U g^{-1} were obtained [13].

We then optimized pectinase production in Erlenmeyer flasks using a 2^3 Rotatable Central Composite Design, where the response variable was pectinase production at 18 h, and the variables analyzed were initial moisture content, $(NH_4)_2SO_4$ concentration, and the ratio of citrus pulp to sugarcane bagasse in the substrate mixture. The optimized values of these variables were 78.4% moisture content (w/w), 3.48% $(NH_4)_2SO_4$ (w/w, based on dry substrate) and 51.6% citrus pulp/48.4% sugarcane bagasse (w/w dry mass in the substrate mixture). This optimization gave a pectinase activity of 30 U g^{-1} in flasks, double that which we had achieved with the previous protocol. A detailed description of this experiment is provided in the supplementary material. This medium and initial moisture content were then maintained throughout the rest of the study.

SSF in the column bioreactor

The study in the column bioreactor was carried out to collect time course data for pectinase activity and respirometry with the new substrate mixture. Fig. 21A shows a lag phase of 7 h, followed by rapid pectinase production, with the activity reaching 45 ± 4 U g^{-1} at 24 h and then remaining constant. The productivity peaked at 1.8 U g^{-1} h^{-1}, between 20 and 24 h. As previously noted for the growth of *A. niger* CH4 on wheat bran [12], the period during which the pectinase activity increased rapidly, 7 to 24 h, also corresponded to the period during which the O_2 uptake rate (OUR) and CO_2 evolution rate (CER) increased rapidly. The maximum OUR was 145 mmol h^{-1} kg^{-1}, obtained at 26 h. Based on a factor of 0.52 J of metabolic heat produced per μmol of O_2 consumed [14], this corresponds to a maximum heat production rate of 21 mW g^{-1} at 26 h. The RQ peaked at a value of 3 at about 10 h, and then declined rapidly, stabilizing around 0.8 after 15 h. The profiles we obtained for RQ and O_2 consumption in the column bioreactor (Fig. 21) are similar to those of Rahardjo et al. [17], who undertook respirometry studies of *A. oryzae* growing on the surface of wheat flour discs.

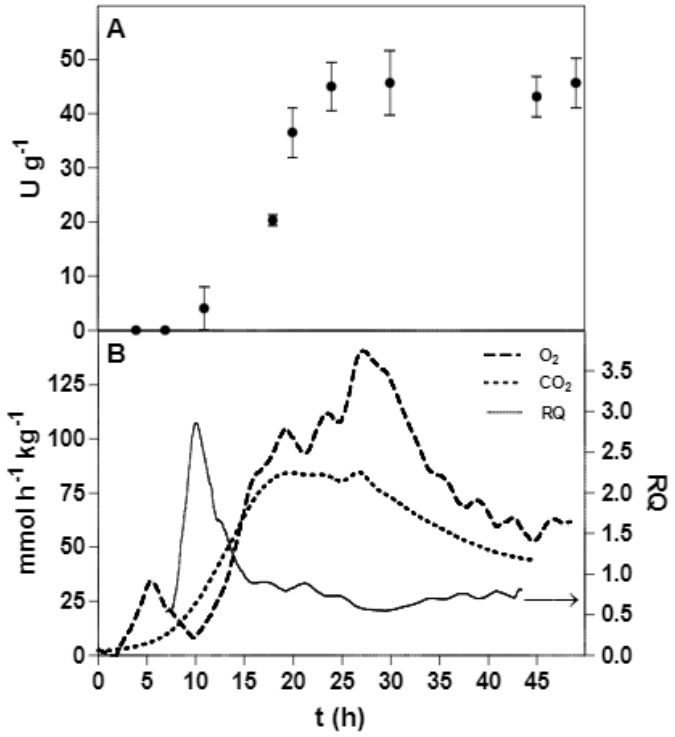

Figure 21. Fermentation in the column bioreactor
(A) Pectinase activities. Results are plotted as the means of triplicates ± the standard error of the mean. (B) Respirometry.

Scale-up of the SSF

The column fermentation was repeated and two pilot-scale fermentations were done. The dimensions of the column bioreactor and pilot bioreactor are compared in Table 6. In all cases, samples were removed at 2- or 3-h intervals and the fermentations were terminated at 24 h. The profile of pectinase activity in this second column fermentation (Fig. 22A) was similar to that obtained in the first (Fig. 21A), but the productivity peaked earlier, at 20 h, with a value of 2.0 U g^{-1} h^{-1}. Again, the period during which the pectinase activity increased (Fig. 22A) overlapped significantly with the period during which the CER increased (Fig. 22D).

Table 6. Dimensions of the laboratory-scale columns and the pilot-scale packed bed bioreactor

	Lab-scale columns	**Pilot-scale**
Bed capacity, dry substrate (kg)	0.008	15
Area of bed cross-section (m^2)	0.001	0.420
Bed height (cm)	12	40
Air flow ($m^3\ h^{-1}$)	0.006	150
Superficial velocity ($m\ s^{-1}$)	0.1	0.1

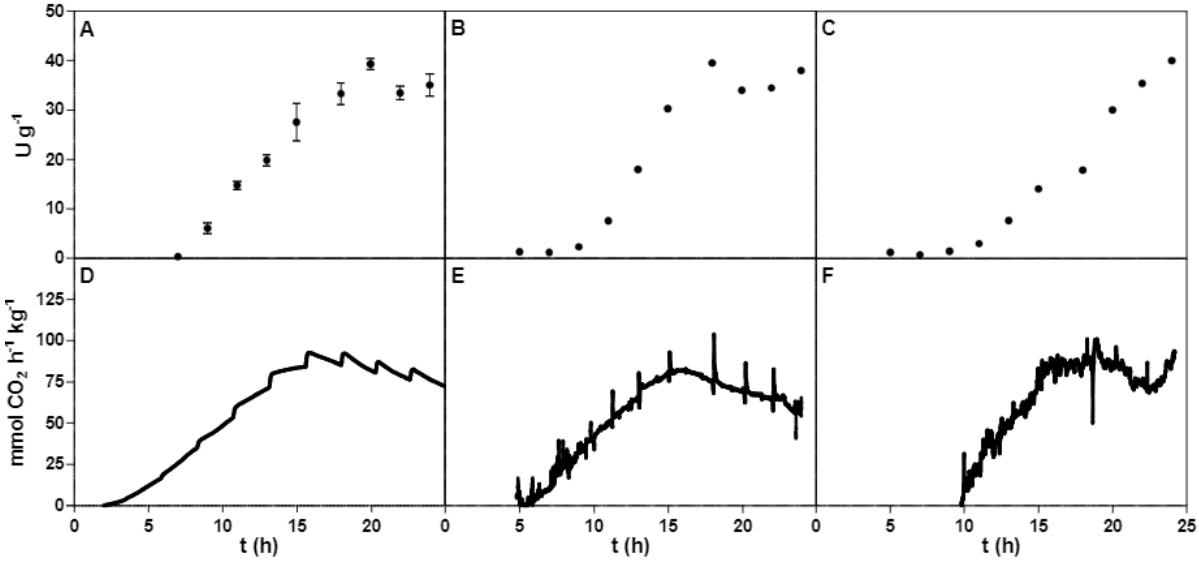

Figure 22. Time courses of the fermentation in the laboratory-scale column bioreactor (first column) the first fermentation in the pilot-scale packed-bed bioreactor (second column) and the second fermentation in the pilot-scale packed-bed bioreactor (third column).
(A), (B) and (C) Pectinase activities. Triplicate samples were removed from different positions of the bed in the column bioreactor (the error bars represent the standard errors of the means), while samples were removed only from the top of the bed in the pilot-scale bioreactor. (D), (E) and (F) CO_2 evolution.

The duplicate fermentations in the pilot-scale bioreactor were undertaken with 15 kg (dry mass) of a mixture of citrus pulp and sugarcane bagasse (51.6/48.4 m/m, dry basis). This gave a bed height of 38-40 cm. Pectinase production was slightly delayed in the pilot-scale bioreactor, with the period of rapid increase starting at around 10 h, whereas this period started at 7 h in the column bioreactor. In all cases, maximum pectinase activities were around 40 U g^{-1}, with these maxima being obtained between 18 and 24 h.

In all three fermentations, the maximum CER values were similar, at around 80 mmol kg^{-1} h^{-1} (Fig. 22, parts D, E and F). However, the peaks were dislocated in time, with the initial increase in the CER beginning at different times and the peaks occurring at different times. The CER profile in the second pilot fermentation started to increase around 5 h later than the other two and also peaked a few hours later.

Although the two pilot fermentations were intended to be duplicates, the air temperature at the bioreactor inlet was about 2°C lower in the second pilot fermentation compared to the first pilot fermentation. This caused the temperature inside the bed during the first 10 h of the second pilot fermentation to be about 2°C lower than that during the first 10 h of the first pilot fermentation (Fig. 23, parts C and D). Notably, in both cases, the temperature within the bed itself was well controlled throughout the fermentation, with all thermocouples registering temperatures within 1°C of the inlet air temperature (Fig. 23, parts C and D). The good temperature control in the bed is not surprising, as the bed maintained a good porosity throughout the fermentations (Fig. 24), without formation of the large agglomerates that were observed by Pitol et al. [12] for the growth of *A. niger* on wheat bran.

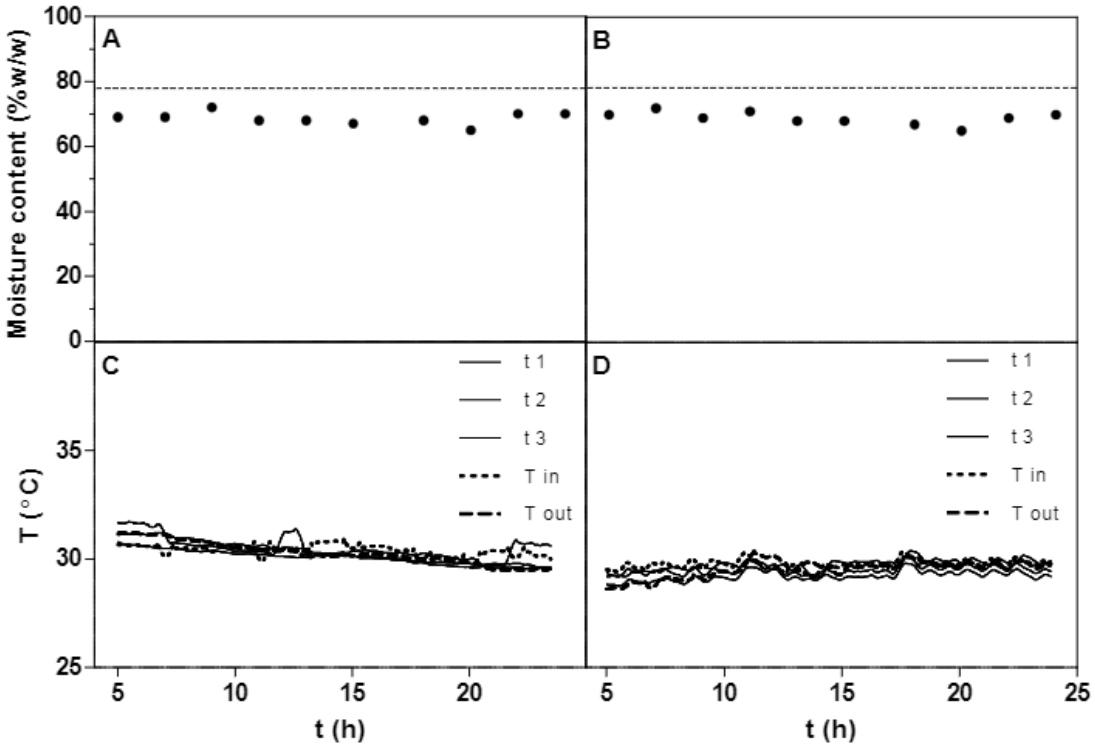

Figure 23. Moisture contents and temperatures in the bed during the pilot-scale fermentations
The first column represents the first pilot-scale fermentation while the second column represents the second pilot-scale fermentation. (A) and (B) Moisture contents at the top of the bed. The dashed lines show the initial moisture content of the bed. (C) and (D) Temperatures measured at three bed heights and for the inlet and outlet air. "T in" is the temperature of the inlet air, "T out" is the temperature of the outlet air and t1, t2 and t3 represent the average temperatures measured by the thermocouples shown in Figure 20.

Figure 24. Fermented solid from the second pilot fermentation, at 24 h

The profiles for moisture content at the top of the bed were quite similar for both pilot fermentations, remaining in the range of 65 to 72% w/w, with more variation with respect to the sampling position than with respect to time (Fig. 23, parts A and B). This absence of drying of the bed along the fermentation is consistent with the absence of a temperature gradient in the bed: since the air is saturated at the air inlet, it can only remove moisture from the bed if high metabolic heat production rates cause it to heat up as it passes through the bed, thereby increasing its water-carrying capacity.

At the end of the two pilot-scale fermentations, the bed was "mapped"; in other words, pectinase activities (Fig. 25A and 25B), and moisture contents (Fig. 25C and 25D) were determined in different positions of the bed. The uniformity of pectinase activity in the bed was different in the two pilot fermentations: in the first pilot fermentation, all pectinase activities were between 33 and 41 U g^{-1} (Fig. 25A), while in the second pilot fermentation they varied from 15 to 42 U g^{-1}, with a slight tendency to be lower at the bottom of the bed (Fig. 25B). In the case of the moisture content, all values fell within the range of 65 to 80%. However, there was a difference: in the first pilot fermentation, there was no trend with height, while in the second pilot fermentation there was a tendency for the moisture content to be higher at lower bed heights. It should be noted that this trend does not imply that evaporation occurred in the bed. Indeed, the moisture content at the top of the bed remained essentially constant throughout the fermentation (Fig. 23B). Rather, it would appear that water was condensing in the bottom of the bed: the mapping shows that the moisture content at the bottom of the bed at the end of the second pilot fermentation was slightly higher than the initial moisture content. These wetter conditions appear to have affected pectinase production negatively.

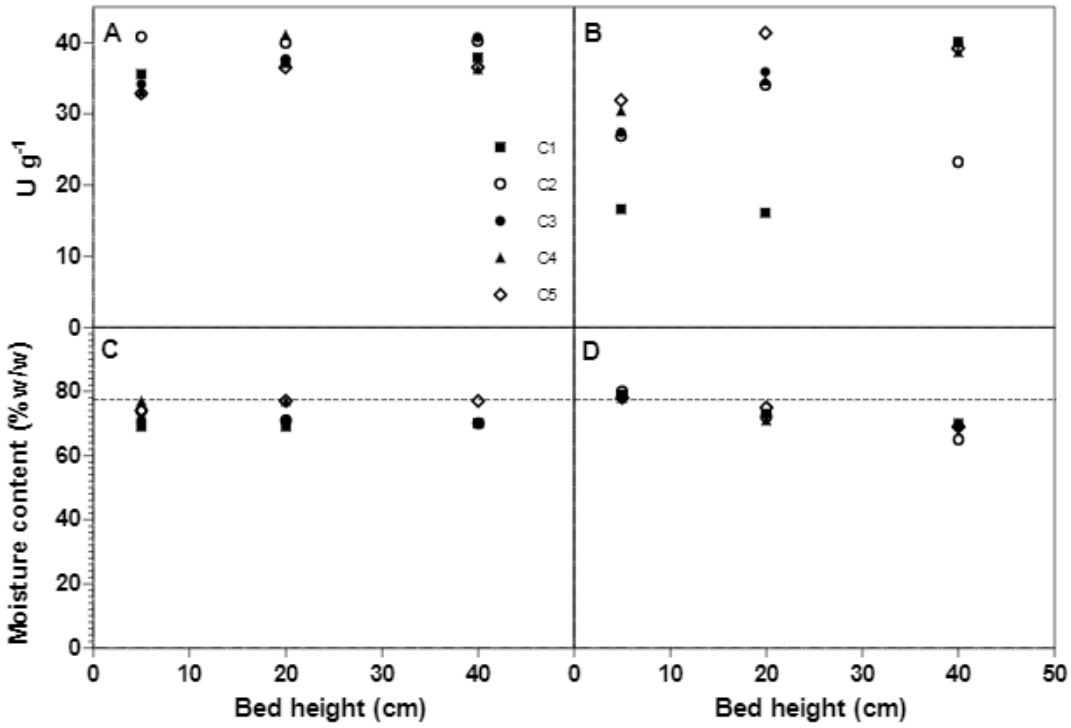

Figure 25. Mapping of the bed within the pilot-scale bioreactor at the end of the fermentation in order to study bed uniformity
The first column represents the mapping of the first pilot fermentation while the second column represents the mapping of the second pilot fermentation. (A) and (B) Pectinase activities at the various sampling positions. (C) and (D) Moisture contents at the various sampling positions. The dashed lines show the initial moisture content of the bed.

Hydrolysis of citric pectin and citrus pulp using the fermented solid as a catalyst

After the second pilot-scale fermentation, fermented solid was removed from several randomly selected positions of the bed, homogenized and then lyophilized. The lyophilized fermented solid (LFS) had an activity of 40 U g^{-1}. It was used in the hydrolysis of citric pectin in two different manners: direct addition of the LFS and addition of an aqueous extract of the LFS. Additionally, a hydrolysis reaction was carried out with a commercial pectinase preparation, Pectinex®. Fig. 26 shows the profiles for liberation of reducing sugars (Fig. 26A) and D-galacturonic acid (Fig. 26B). It is noticeable that direct addition of the LFS and addition of an aqueous extract gave results quite similar to those obtained with Pectinex®.

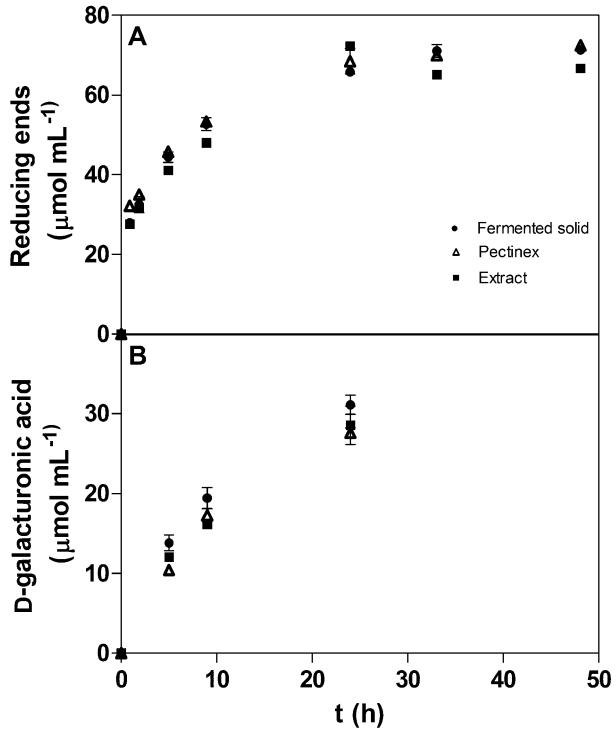

Figure 26. Hydrolysis profile of 2% citrus pectin using the fermented solid and the extract obtained from the second pilot fermentation, as well as the commercial pectinase preparation Pectinex®
(A) Release of reducing sugars assayed by the DNS method. (B) D-galacturonic acid production, determined by HPLC. The error bars represent the standard errors of the means from three different experiments.

Discussion

In this work, we scaled up an SSF process for the production of fermented solids with pectinolytic activity and showed that these solids can be used to liberate reducing sugars from pectin-rich substrates. Our pilot-scale fermentation, with growth of *Aspergillus oryzae* on 15 kg of a 51.6:48.4 mixture (m/m, dry basis) of citrus pulp and sugarcane bagasse, showed a superior performance to that of Pitol et al. [12], who grew *Aspergillus niger* on 30 kg of a 90:10 mixture (dry basis) of wheat bran and sugarcane bagasse in the same pilot-scale bioreactor. In the first pilot fermentation, in which condensation of water in the base of the bed was avoided, we produced pectinase activities around 37 U g^{-1} (i.e., from 33 to 41 U g^{-1}) uniformly across the bed, whereas the best result of Pitol et al. [12] for the average pectinase activity within the bed was 20 U g^{-1}, with poor uniformity of pectinase activity across the bed in most of their pilot-scale fermentations. Although our solid substrate mixture has a lower bulk density than

that used by Pitol et al. [12], such that the loading of the bed is much lower, the overall amount of pectinase activity that we produced in the bioreactor was similar: they obtained 20 U g^{-1} with 30 kg dry substrate, giving an overall amount of 600 × 10^3 U in the bioreactor; we obtained 37 U g^{-1} with 15 kg dry substrate (in the second fermentation), giving an overall amount of 555 × 10^3 U in the bioreactor. As we discuss below, our system has better bed properties, which confer on it a better potential for further scale-up.

By using a much higher content of sugarcane bagasse than did Pitol et al. [12], 48.4% compared to only 10%, we not only ensured a higher porosity within our bed, but we also provided a more stable bed structure. As a result, we managed to avoid two critical problems reported by Pitol et al. [12]: the formation of compact agglomerates of wheat bran particles, with consequent localized overheating within the agglomerates, and bed shrinkage, which resulted in the formation of preferential flow paths and generalized overheating of the bed. In some cases, they measured temperatures as high as 47°C, compared to an inlet air temperature of 32°C. With the better bed structure, our system has the potential to be used with greater bed heights than the 40 cm used by Pitol et al. [12]. In fact, it is notable that the addition of 10% sugarcane bagasse allowed them to obtain uniform pectinase production with a 30-cm bed height in their second pilot fermentation, but when they increased the bed height to 40 cm, the problems with bed shrinkage returned. Additionally, we can control bed temperatures using a single water reservoir, held at the optimum temperature for growth, to supply water to the humidification column. As a result, our air preparation system is simpler than that of Pitol et al. [12], who, in their fourth pilot fermentation, controlled the temperature of the inlet air by switching between two water reservoirs held at different temperatures.

Our bioreactor is also significantly simpler to construct than the tray bioreactor system with pressure pulsation that was used for the production of pectinases in SSF by He and Chen [11]. Since they cycle the pressure within the tray chamber to gauge pressures as high as 1.5 atm, this chamber needs to be built to the specifications of a pressure vessel and a compressor is required to achieve these high pressures. Our bioreactor operates at a gauge pressure close to zero, since the pressure drop through the bed is only a few centimeters of water, such that the bioreactor is much cheaper to construct and a blower can be used for aeration of the bed. Additionally, our bioreactor system is more compact than theirs: although both our process and theirs involve 15 kg of dry substrate, their tray chamber has a total volume of 800 L while ours

has a total volume of 400 L. The construction of our bioreactor is also considerably simpler than that of Huerta et al. [10], who produced pectinases using a so-called “absorbed-substrate fermentation”, in which a nutrient solution was absorbed onto sugarcane bagasse. They used a Zymotis-type packed bed, which has a system of closely spaced heat transfer plates within the bed.

Our fermentation could be scaled up further following the suggestion of Pitol et al. [12], which was to maintain a relatively low bed height and increase scale by increasing the width to several meters, while fixing the superficial air velocity at a constant value. Such “low-wide packed-beds” are used in the production of soy sauce *koji* [18]. Given the problems that Pitol et al. [12] encountered with lack of uniformity, they suggested maintaining a 40-cm bed height. Considering the better behavior of our bed, it might be possible for us to increase the bed height beyond 40 cm in such a scale-up procedure, although further work must be done to confirm this.

Our proof-of-concept experiment, in which we hydrolyzed pectin successfully with the direct addition of lyophilized fermented solids into the reaction mixture, is promising for a biorefinery: This strategy reduces costs by avoiding the need for enzyme extraction and concentration. It has been demonstrated successfully with lipases produced by SSF [19, 20]. In the case of lipases, it was possible to replace the lyophilization with a simple air drying [21], by passing dry air through the bed at the end of the fermentation. This possibility needs to be tested with the fermented solids produced in the current work.

Conclusions

We have shown that pectinases can be produced successfully in a pilot-scale packed-bed SSF bioreactor, using a medium based on 51.6% citrus pulp, to induce pectinase production, and 48.4% sugarcane bagasse, to ensure a high bed porosity and stable bed structure. This solid substrate mixture avoided the problems of formation of agglomerates and bed shrinkage that were reported in the earlier work of Pitol et al. [12] for the production of pectinases, in the same bioreactor, with a medium composed primarily of wheat bran (90%), with a relatively small content of sugarcane bagasse (10%). As a result, the bed temperature within our bioreactor was

well controlled, and pectinase production was reasonably uniform across the bed. This system has good potential for scaling up to larger scales.

Acknowledgements

This research was supported by a "Universal" grant from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), a Brazilian government agency for the advancement of science and technology. Research scholarships were granted to David Mitchell and Nadia Krieger by CNPq, to Bruna Medina by Fundação Araucária, an agency for the support of science and technology in the state of Paraná, and to Luana Oliveira Pitol, Anelize Finkler and Alessandra Biz by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), a Brazilian government agency for the development of personnel in higher education.

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Supplementary material for
"Production of pectinases by solid-state fermentation on a mixture of citrus waste and sugarcane bagasse in a pilot-scale packed-bed bioreactor"

by

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This section presents the optimization study carried out to improve the production of pectinases in solid-state fermentation (SSF) by *Aspergillus oryzae* on a mixture of sugar cane bagasse and citrus pulp. A rotatable central composite design (RCCD) was used. The experiments were performed in Erlenmeyer flasks and with fixed fermentation time. The central values of the variables and the fermentation time were chosen based on previous experience of our laboratory with pectinase production using this strain and substrate mixture.

Materials and Methods

Microorganism

The strain used was *Aspergillus oryzae* CPQBA 394-12 DRM 01, originally isolated from decomposing passion fruit peels and identified by CPQBA–UNICAMP (Campinas, Brazil). To prepare the spore suspension, the strain was grown in potato dextrose agar for 3-5 days and the spores were extracted with water. The suspension was filtered through gauze. The spore concentration in the suspension was determined using a Neubauer chamber.

Substrates

Sugarcane bagasse and citrus pulp were kindly donated by Usina de Álcool Melhoramentos (Jussara, Brazil) and Corol Cooperativa Agroindustrial (Rolândia, Brazil), respectively.

Rotatable Central Composite Design (RCCD)

Table 7 shows the independent variables and the values used within the RCCD experiment. The response variable was pectinase activity (U per gram of total dry solid) at 18 h.

Table 7. Variables and levels

Variable	**Code**	**Levels used**				
(units)		**-1.68**	**-1**	**0**	**+1**	**+1.68**
X_1: Initial moisture content (% w/w, wet basis)	X_1	61.6	65	70	75	78.4
X_2: $(NH_4)_2SO_4$ (% w/w, based on total dry substrate)	X_2	2.55	3.3	4.4	5.5	6.25
X_3: citrus pulp (% w/w, based on total dry substrate	X_3	51.6	55	60	65	68.4

There were 17 experiments, corresponding to a full 2^3 factorial design, with six additional axial points (i.e. at coded values of +1.68 and -1.68) and three replicates of the central point (Table 8). Each experiment was performed in duplicate and the mean of the response was used in the calculations. The results were analysed using Statistica 10 (Statsoft^{TM}), where the effects of the variables and respective errors were calculated at a 95% confidence level. An analysis of variance (ANOVA) was done and the values of F from the Fisher test were used to determine the significance of the model. The contour plots and the response surfaces were obtained with the definition of the optimal operating ranges for each level.

Fermentation procedure

The moisture content was determined in an infrared balance (Gehaka IV 2000, São Paulo, Brazil) and reported on a wet basis. In each experiment, 5 g of the substrate mixture (dry basis), with the proportions of citrus pulp and sugarcane bagasse determined by the statistical planning, were autoclaved (121°C, 15 min). An $(NH_4)_2SO_4$ solution was also autoclaved (121°C, 15 min) and added to the flask, to give the initial moisture content (wet basis) and $(NH_4)_2SO_4$ concentration dictated by the statistical planning (w/w of the substrate, dry basis). A spore suspension was added to the moist substrate to obtain 5×10^7 spores per gram of dry substrate and mixed in with sterile glass rod. The water in the spore suspension was taken into account to obtain the correct initial moisture content. Flasks were incubated at 30°C for 18 h.

Table 8. Matrix of the RCCD planning

Experiment	X_1	X_2	X_3
1	+1	-1	-1
2	-1	-1	-1
3	+1	+1	-1
4	-1	+1	-1
5	+1	-1	+1
6	-1	-1	+1
7	+1	+1	+1
8	-1	+1	+1
9	-1.68	0	0
10	+1.68	0	0
11	0	-1.68	0
12	0	+1.68	0
13	0	0	-1.68
14	0	0	+1.68
15	0	0	0
16	0	0	0
17	0	0	0

Pectinase activity determination

100 mL of acetate buffer (0.2 M, pH 4.5) was added to each Erlenmeyer. The extraction was carried out at 160 rpm and 30°C for 30 min. The crude extracts were filtered through gauze and diluted 1:5 in the same acetate buffer. For the pectinase assays, 0.25 mL of crude extract was incubated with 0.25 mL of 1% (w/v) citric pectin (Sigma, 75% methylation) in acetate buffer (0.2 M, pH 4.5) for 20 min at 30ºC. A standard curve of D-galacturonic acid (Sigma-Aldrich, ≥ 98.0% purity) was used. The release of reducing sugars was analysed using the DNS method [1]. Pectinase activities were expressed in μmol of reducing ends released per minute, on the basis of the total mass of dry substrate (i.e. U g^{-1}).

Results

The pectinolytic activities obtained varied from 0.6 to 28.1 U g^{-1} and the activities predicted by the model were similar to the experimental values (Table 9).

Table 9. RCCD matrix, showing the real and coded values of the independent variables as well as the experimental and predicted pectinolytic activities

Experiment	X_1 Moisture (% w/w)*	X_2 $(NH_4)_2SO_4$ (% w/w) #	X_3 citrus pulp (%w/w) #	1st replicate (U g^{-1})	2nd replicate (U g^{-1})	Mean (U g^{-1})	Activity predicted by the model (U g^{-1})
1	65 (-1)	3.3 (-1)	55 (-1)	11.3	14.4	12.85	10.6
2	75 (1)	3.3 (-1)	55 (-1)	22.2	17.5	19.85	22.7
3	65 (-1)	5.5 (1)	55 (-1)	8.8	6.8	7.8	8.6
4	75 (1)	5.5 (1)	55 (-1)	24.5	23.6	24.05	20.7
5	65 (-1)	3.3 (-1)	65 (1)	5.5	6.0	5.75	5.3
6	75 (1)	3.3 (-1)	65 (1)	16.8	17.5	17.15	17.3
7	65 (-1)	5.5 (1)	65 (1)	2.6	3.6	3.1	3.2
8	75 (1)	5.5 (1)	65 (1)	14.6	14.2	14.4	15.3
9	61.6 (-1.68)	4.4 (0)	60 (0)	0.6	7.7	4.15	2.9
10	78.4 (+1.68)	4.4 (0)	60 (0)	23.4	28.1	25.75	23.1
11	70 (0)	2.55 (-1.68)	60 (0)	16.2	14.0	15.1	14.7
12	70 (0)	6.25 (+1.68)	60 (0)	10.6	10.5	10.55	11.3
13	70 (0)	4.4 (0)	51.6 (-1.68)	14.0	15.7	14.85	17.5
14	70 (0)	4.4 (0)	68.4 (+1.68)	8.0	6.9	7.45	8.5
15	70 (0)	4.4 (0)	60 (0)	8.6	16.7	12.65	13.0
16	70 (0)	4.4 (0)	60 (0)	12.4	15.9	14.15	13.0
17	70 (0)	4.4 (0)	60 (0)	11.8	10.0	10.9	13.0

*wet basis; # dry basis

The Pareto chart (Fig. 27) shows that only the linear effects were significant at the level of 5 % ($p < 0.05$). The initial moisture content had a strong positive effect on the pectinolytic activity. The $(NH_4)_2SO_4$ concentration and the percentage of citrus pulp had negative effects, meaning that higher levels correspond to lower pectinolytic activities.

The following linear equation was obtained after the elimination of the non-significant parameters:

$$\mathbf{Y} = -35.12 + 1.20 \times \mathbf{X_1} - 0.92 \times \mathbf{X_2} - 0.54 \times \mathbf{X_3}$$

where Y is the pectinolytic activity (U g^{-1}) and X_1, X_2, and X_3 are the coded values for the initial moisture content, the $(NH_4)_2SO_4$ concentration and the percentage of citrus pulp, respectively.

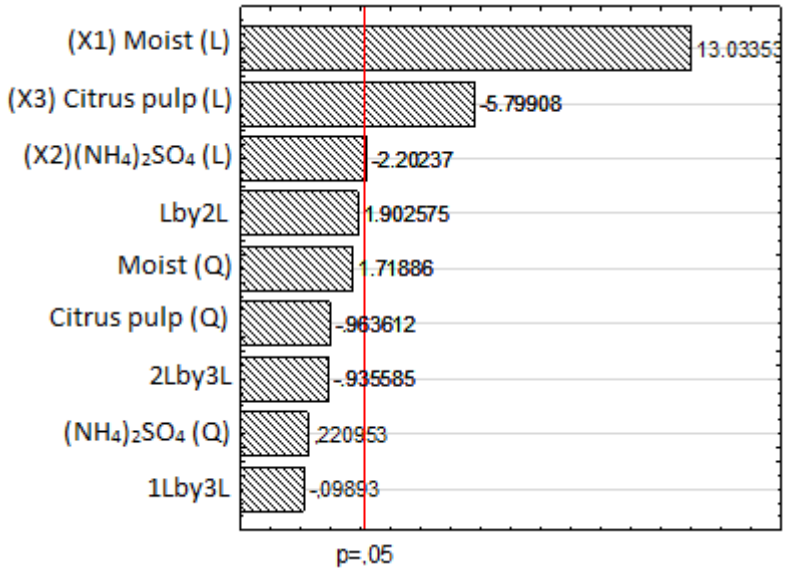

Figure 27. Pareto chart analysis of linear (L) and quadratic (Q) effects. Absolute values

The residues are distributed randomly (Fig. 28A). In the plot of observed versus predicted values (Fig. 28B), the data points are close to the diagonal line, indicating a good fit of the model to the experimental data.

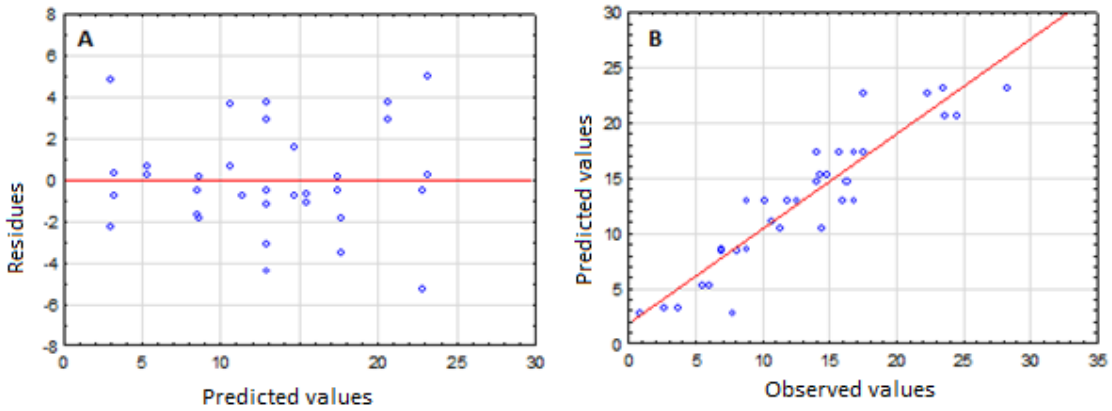

Figure 28. (A) Distribution of residues. (B) Observed versus predicted values

The analysis of variance (ANOVA) (Table 10) shows that the model is significant at the level of 5 % ($p < 0.05$), since the calculated F-value is greater than the critical F-value. Additionally, the coefficient of determination (R^2) obtained for the model is 0.86, showing that the model explains 86% of the variance in the response.

Table 10. Analysis of variance (ANOVA) of the model

Factors	**Sum of squares**	**DF***	**Quadratic mean**	**F-value CALC**	**TAB**	***p*-value**
X_1: moisture (L)	989.872	1	989.872			0.000000
X_2: $(NH_4)_2SO_4$ (L)	28.264	1	28.264			0.040189
X_3: citrus pulp (L)	195.962	1	195.962			0.000014
Regression (R)	1214.0984	3	404.699	61.023	2.92*	
Residues (r)	198.9566	30	6.632			
Lack of fit (LF)	88.241	11	8.022	1.063	2.34*	0.411213
Pure error	110.716	19	5.827			
Total	1413.055	33	-			
R^2	0.859	-	-			

* Degrees of freedom

Since the model was predictive and significant, we plotted response surfaces and contour plots (Fig. 29). The optimal conditions for pectinase production predicted by the model were an initial moisture content of 78.4 % (w/w, wet basis), an $(NH_4)_2SO_4$ concentration of 3.48 % (w/w, dry substrate) and 51.6 % of citrus pulp and 48.4% sugarcane bagasse in the substrate mixture. Under these conditions, the model predicted a pectinase activity at 18 h of 28.4 U g^{-1}. An experiment was carried out in quintuplicate under the optimized conditions to validate the model. The pectinolytic activity obtained was 30 ± 1 U g^{-1} (mean ± standard error of the mean). This observed value is double the value obtained with the previous, non-optimized, fermentations (15 ± 1 U g^{-1}) [2], and is close to the value of 28.4 U g^{-1} predicted by the model.

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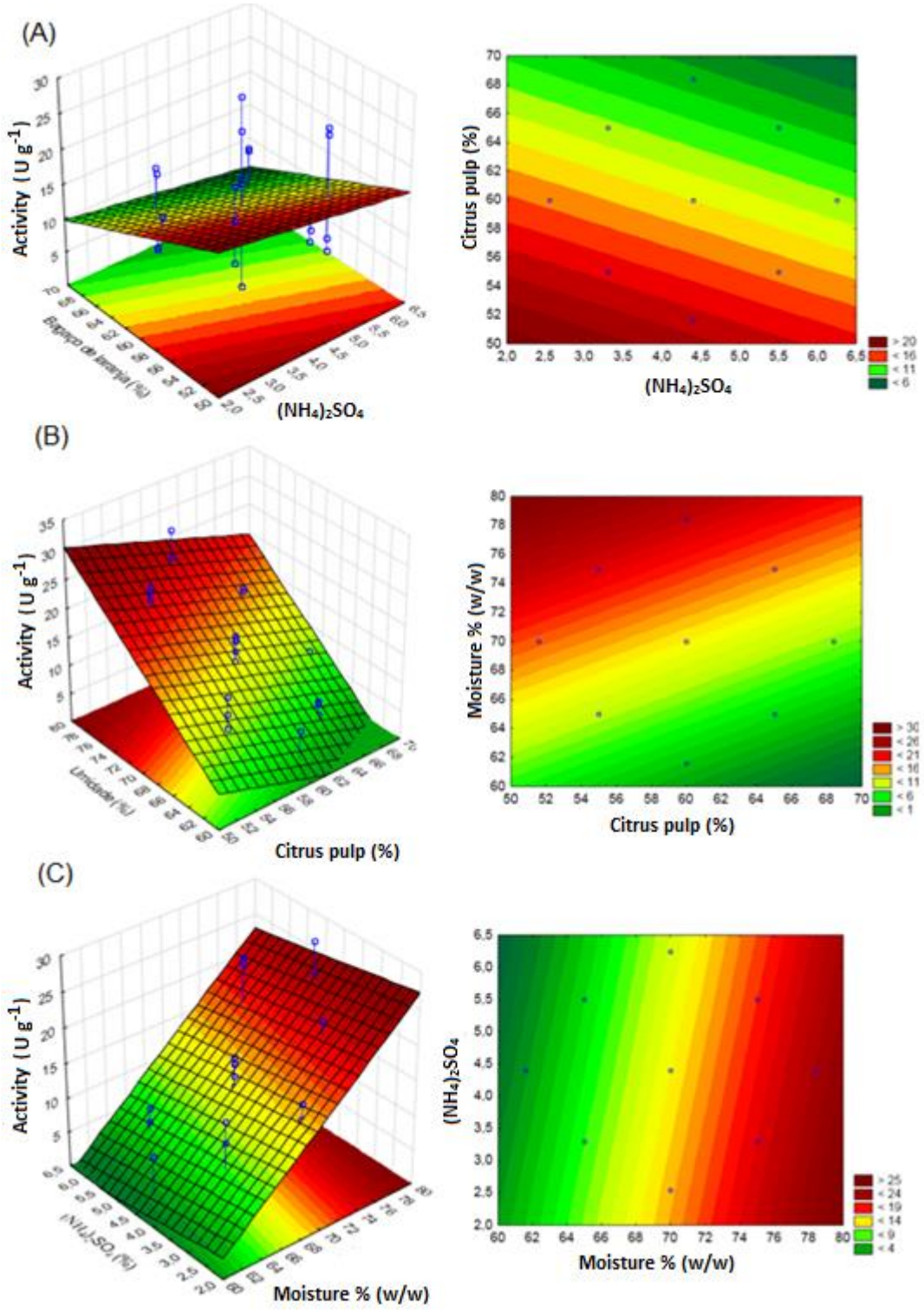

Figure 29. Surface plots (left) and contour plots (right)
(A) Percentage of citrus pulp and $(NH_4)_2SO_4$ concentration. (B) Initial moisture content and percentage of citrus pulp. (C) Initial moisture content and $(NH_4)_2SO_4$ concentration. In all the surfaces, the value of the third variable was maintained in the central point.

Capítulo 4

Manuscrito do artigo 3

Construction of a strain of *Saccharomyces cerevisiae* that consumes D-galacturonic acid with the integration of the fungal reductive pathway

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ABSTRACT

Pectin-rich wastes are produced in considerable amounts in several countries and could be used as raw materials for biorefineries. One possible process in such biorefineries is the production of ethanol from hydrolysates rich in D-galacturonic acid. However, the ethanol-producing organism of choice, *Saccharomyces cerevisiae*, is not able to catabolize D-galacturonic acid. In the current work, we describe the construction of a strain of *S. cerevisiae* in which the four genes of the fungal reductive pathway for D-galacturonic acid catabolism were integrated into the genome: *gaaC*, *gaaD* and *gaaA* from *Aspergillus niger* and *lgd1* from *Trichoderma reesei*. This strain consumed D-galacturonic in a medium containing fructose. This represents the first step in the construction of a strain of *S. cerevisiae* that would be able to produce ethanol from D-galacturonic acid.

Keywords: *Ethanol, D-galacturonic acid, Saccharomyces cerevisiae, reductive pathway, citrus pulp, metabolic engineering*

Introduction

Citrus pulp and sugar beet pulp are pectin-rich wastes that are produced in considerable amounts in various countries. Citrus pulp results from the production of frozen orange juice concentrate. Its production is concentrated mainly in Brazil and the USA, which detain 68% and 32% of the global market, respectively [1]. In the 2014/2015 harvest, 1.4 million tons of orange juice (65°Brix) were produced in these two countries [1], and this amount would result in the production of about 1.9 million metric tons of citrus pulp (dry matter). Likewise, large amounts of sugar beet pulp are generated during the extraction of sugar from sugar beets in temperate countries, especially Russia, the USA and several European countries [2]. In the 2013 harvest, a total of 246 million metric tons of sugar beet were produced worldwide [2], resulting in 12.3 million metric tons of beet pulp.

Citrus pulp and sugar beet pulp are usually sold for incorporation in cattle feed, but the costs of drying these wastes makes this barely profitable [3]. On the other hand, these wastes are potentially important sources of carbohydrates that can be used as raw materials in biorefineries

for the production of bio-based chemicals and biofuels. In fact, there is a rising demand for ethanol, especially in Brazil and the USA, and biorefineries using citrus wastes or sugar beet pulp could potentially meet part of this demand [4,5].

Hydrolysates obtained from pectin-rich wastes have high contents of galacturonic acid, which represents 18% (w/w) of citrus waste hydrolysates [6] and 16 % (w/w) of sugar beet pulp hydrolysates [7]. Unfortunately, this acid sugar is not fermentable to ethanol by *Saccharomyces cerevisiae*, which is the microorganism of choice for ethanol production. Conversely, many strains that are able to use galacturonic acid do not produce ethanol in appreciable amounts. *Escherichia coli*, for example, has the isomerase pathway for galacturonic acid catabolism, but, due to its mixed acid fermentation, the production of ethanol is very low. Ohta *et al*. [8] increased ethanol production by cloning two genes of *Zymomonas mobilis*, alcohol dehydrogenase (*adhB*) and pyruvate decarboxylase (*pdc*), into *E. coli* K011. After selection of mutants, they obtained an ethanol concentration of 3.86 g L^{-1} when 20 g/L galacturonic acid was used as the sole carbon source [9]. Such levels are not sufficiently high to warrant using this strain for commercial ethanol production.

On the other hand, ethanol production with *S. cerevisiae* is already a highly productive and robust industrial process. This yeast is preferred for commercial ethanol production for several reasons. Unlike most bacteria, *S. cerevisiae* is resistant to the unfavorable conditions that are encountered in industrial processes, such as the presence of contaminants, bacteriophages and inhibitors, and low pH [10]. It also tolerates higher osmotic pressures, enabling the use of a concentrated culture medium, and greater concentrations of ethanol. This improves the economic viability of ethanol plants, because the bioreactors are smaller and the amount of water entering the distillation process is correspondingly smaller [10]. Considering this, the cloning of a heterologous pathway for catabolism of D-galacturonic acid into *S. cerevisiae*, instead of cloning genes for ethanol production into bacteria, has more chance of enabling industrial ethanol production from hydrolysates containing D-galacturonic acid.

In this work, we integrated five genes of the reductive galacturonic catabolic pathway from three different fungi, *Aspergillus niger, Trichoderma reesei and Neurospora crassa*, into the genome of *S. cerevisiae*, in order to make a GMO yeast that is able to use galacturonic acid as

a carbon source. This work represents a preliminary step in the construction of a strain of *S. cerevisiae* able to produce ethanol from D-galacturonic acid.

Materials and Methods

Strains

The strain H2811 (*S. cerevisiae* CNPK11314C *ΔHIS3*, *ΔLEU2*, *ΔURA3*) was used as a host for the integration of the genes. This strain was also used as the wild type control for the fermentation trials. All the plasmids were produced in *E. coli* TOP10 cells and the homologous recombinations for the construction of plasmids were carried out in *S. cerevisiae* strain ATCC 90845 (H3488). Table 11 shows the original and engineered *S. cerevisiae* strains used in this work.

Table 11. Strains used in this work

Strains	Description
Escherichia coli TOP10	*E. coli* for electroporation *hsdR, mcrA, lacZΔM15, recA*
H2811	*S. cerevisiae* CNPK11314C *ΔHIS3*, *ΔLEU2*, *ΔURA3*
H3488	*MATα, his3Δ 200, ura3-52, leu2 Δ 1, lys2 Δ 202, trp1 Δ 63* for homologous recombination in yeast
A. niger ATCC 1015	template DNA for gene *gaaC*
Schizosaccharomyces pombe	template DNA for gene *his5*
H4362	*S. cerevisiae* CNPK11314C (H2811) expressing the gene *gaaC* integrated into the locus *HIS3* in the yeast genome
H4410	*S. cerevisiae* CNPK11314C + *gaaC* (H4362), expressing the gene *gaaD* integrated into the locus *LEU2* in the yeast genome
H4425	*S. cerevisiae* CNPK11314C + *gaaC* + *gaaD* (H4410), expressing the codon optimized gene *gaaA** integrated into the locus *URA3* in the yeast genome
H4531	*S. cerevisiae* CNPK11314C + *gaaC* + *gad* + *gaaA** (H4425), expressing the gene *lgd1* integrated into the locus *CAN1* in the yeast genome
H4535	*S. cerevisiae* CNPK11314C + *gaaC* + *gad* + *gaaA** + *lgd1* (H4531), expressing the gene *gat1-gfp* integrated into the locus *HO* in the yeast genome

Plasmid construction and gene integrations

Plasmids are listed in Table 12. Primers used for plasmid construction are listed in Table 13. All the amplifications were performed using Phusion High Fidelity (Finnzymes, Finland) and

Phusion High Fidelity buffer or GC Buffer (Finnzymes, Finland). For bacterial and yeast colony PCR DyNAzyme (Finnzymes, Finland) was used instead. For yeast colony PCR, cell disruption was carried out using Zymolyase 100 T (Seikagaku Biobusiness, Japan). All the amplifications were done with Bioer XP (Bioer Technology CO. Ltd., China). PCR products and plasmideal DNA were purified using QIAprep Spin Miniprep Kit (Qiagen GmbH, Germany). All the digestions were made using 1× Fast Digest buffer (Thermo Scientific, USA). Linear DNA fragments were isolated from 1% (w/v) agarose gel in 1× TBE buffer using QIAquick Gel Extraction Kit (Qiagen GmbH, Germany). Dephosphorylations were done using Antarctic Phosphatase (New England Biolab, USA) and ligations were done with T4 DNA ligase (Promega, USA). Recombination in yeast for plasmid assembly or genome integration was done using Lab Transformation kit (Molecular Research Reagents Inc., USA), following the protocol of Gietz et al. [11]. Recombination *in vitro* was done using Gibson Assembly® Master Mix (New England Biolabs, USA). Sequencing was done out of company by Gatc Biotech (Germany).

Table 12. Plasmids used in this work

Plasmid	Description
B1181	YEplac195 with *PGK1* promoter, *URA3*, Amp^R
pRS426	yeast integration vector, *URA3*, Amp^R
B5430	B1181 ligated to *gaaC*. Contains fragment 3 *(PGK1*/*gaaC)* of the *gaaC* integration vector in between *bglII* restriction sites
B5517	yeast integration vector created by homologous recombination in yeast. Contains the gene *gaaC* under *PGK1 promoter*, the integration *locus HIS3* and marker *HIS5* (*S. pombae*) in between *NotI* restriction sites
B5470	pXY212 expressing the gene *gaaD* under *TPI1* promoter
pRS405	yeast integration vector, *LEU2*, Amp^R
B5555	yeast integration vector pRS405 expressing *LEU2* and *gaaD* under *TPI1* promoter in between *AflII* restriction sites
B2159	pXY212, containing *TPI1* promoter region, Amp^R
B5706	Genescript pUC57 plasmid containing codon optimized *gaaA** in between *EcoRI* and *BamHI* restriction sites
B5696	B2159 ligated to *gaaA** under *TPI* promoter
pRS406	yeast integration vector containing *URA3*, Amp^R
B5697	yeast integration vector containing *URA3* and *gaaA** under *TPI1* promoter in between *AflII* restriction sites
B3033	yeast integration vector containing *CAN1* l*ocus*, *KnMX* and *lgd1* under *PGK1* promoter in between LoxP sites and *SaclL* and *Kpnl* restriction sites
pSH66	deletion vector containing *Cre*, Amp^R
B6367	B2159 containing *gat1-gfp* under *TPI1* promoter
Vector HO	yeast integration vector containing *KnMX* marker, *HO* locus
B6382	Vector HO containing *gat1-gfp* under *TPI1* promoter

Table 13. Primers used in this work

Primer	Sequence	Description
P1	ATGCCTTTTACCCCGCTCCG	for *gaaC* amplification from *A. niger* genome, for colony PCR and sequencing (forward)
P2	CTAAGCAATATCCGGCAACG	for *gaaC* amplification from *A. niger* genome, for colony PCR and sequencing (reverse)
P3	CGGGGGATCCACTAGTTCTAGAGCG GCCGCGTGAGGGTCAGTTATTTCAT	for fragment 1 (-1000 bp HIS3 locus) amplification from *S. cerevisiae* (forward)
P4	TATTTCTTTCTACAAAAGCCCTCCTA CCCATCTTTGCCTTCGTTTATCTTG	for fragment 1 (-1000 bp HIS3 locus) amplification from *S. cerevisiae* (reverse)
P5	TAACTCGAAAATTCTGCGTTCGTTA AAGCTAGCTGCAGCATACGATATAT	for fragment 4 (+1000 bp *HIS3* locus) amplification from *S. cerevisiae* (forward)
P6	AAGCTGGAGCTCCACCGCGGTGGCG GCCGCGGAGCCATAATGACAGCAGT	for fragment 4 (+1000 bp *HIS3* locus) amplification from *S. cerevisiae* (reverse)
P7	AATGAGCAGGCAAGATAAACGAAG GCAAAGATGGGTAGGAGGGCTTTTG T	for fragment 2 (*his5*) amplification from *S. pombae* (forward)
P8	TTCAGTTTTGGATAGATCAGTTAGA AAGCTATTAAGGGTTCTCGAGAGCT	for fragment 2 (*his5*) amplification from *S. pombae* (forward)
P9	GGAAGATATGATCTACGTATGGTCA TTTCTTC	for *TPI1*/*gaaD* amplification from B5470 (forward)
P10	GGAGATCTCGAATTGGAGCTAGAGA AAG	for *TPI1*/*gaaD* amplification from B5470 (reverse)
P11	GATCTACGTATGGTCATTTCTTC	for colony PCR and sequencing *gaaD* ORF (forward)
P12	TCGAATTGGAGCTAGACAAAG	for colony PCR and sequencing *gaaD* ORF (reverse)
P13	ATGGCTCCCCCAGCTGTGTT	for colony PCR and sequencing *gaaA** ORF (forward)
P14	CTACTTCAGCTCCCACTTTC	for colony PCR and sequencing *gaaA** ORF (reverse)
P15	CCTCGCACCCATGTACATTGG	for colony PCR and sequencing *gat1* ORF (forward)
P16	TTATATTGGCCTTTATGTCCGC	for colony PCR and sequencing *gat1* ORF (reverse)
P17	TATATACCCGGGGTGCCACCTGACG TCTAAGA	for amplification of *TPI1*/*gat1-gfp* from B6367 (forward)
P18	TATATACCCGGGAGACCGAGATAGG GTTGAGT	for amplification of *TPI1*/*gat1-gfp* from B6367 (reverse)

gaaC integration

The *gaaC* integration cassette was designed to have the *gaaC* gene under the *PGK1* promoter (fragment 3), the flanking regions of *S. cerevisiae his3* gene (fragments 1 and 4), a homologous region for genome integration, and the *S. pombae his5* gene (yeast marker, fragment 2) assembled circularly in the pRS426 vector (fragment 5), which contains a *ura3* marker. The *ura3* marker was used for the selection of the assembled plasmid and the *his5* marker was used for selection of the *gaaC*-integrated H2811, as it is auxotrophic for *his3*.

As *gaaC* is free from introns, it was directly amplified from the *A. niger* ATCC 1015 genome using the primers P1 and P2. These primers had a 20 bp region homologous to plasmid B1181, thus, *gaaC* was inserted into the *PGK1* (phosphoglicerate kinase) promoter region using Gibson Assembly. The resulting plasmid, B5430, contains the *PGK1/gaaC* component (fragment 3) of the *gaaC* integration cassette. B5430 was digested with *BglII* to obtain fragment 3. Fragment 1, which corresponds to the -1000 bp downstream region of *S. cerevisiae his3*, and fragment 4, which corresponds to the upstream +1000 bp region of *his3*, were amplified from the *S. cerevisiae* (H3488) genome. The primers P3/P4 were used for the amplification of fragment 1. P3 has a 20 bp region homologous to the pRS426 vector (fragment 5, *ura3* marker) and a *Not1* restriction site. P4 has a 20 bp region homologous to *his5* (fragment 2). The primers P5/P6 were used for the amplification of fragment 4. P5 has a 20 bp region homologous to fragment 3. P6 has a 20 bp region homologous to the pRS426 vector and a *NotI* restriction site. *his5* (fragment 2) was amplified from the *S. pombae* genome using P7 and P8 primers. P7 has a 20 bp region homologous to fragment 1 and P7 has a 20 bp region homologous to fragment 3. The pRS426 vector was linearized by digestion with *NotI* (fragment 5). All the fragments were run in an electrophoresis gel for size determination and purification. The recombination in yeast was done using the Gietz protocol. The transformants were selected in SCD - URA plates (synthetic complete medium, without uracyl, supplemented with 20 g D-glucose), re-streaked in another SCD - URA plate for plasmid rescue and preselected by colony PCR for the *gaaC* ORF by using P9 and P10 primers. The assembled plasmid was eletroporated into *E. coli* and the sequence was confirmed by sequencing fragments 1 to 4. Cells from a colony carrying the correct plasmid were stored as B5517.

B5517 was digested with *NotI* for the selection of the integration cassette, which consisted of the assembled fragments 1 to 4. The integration of the *gaaC* cassette into the parental strain H2811 was done by recombination using the Gietz protocol. Transformants were selected in SCD – HIS (synthetic complete medium, without histidine, supplemented with 20 g D-glucose) and confirmed by colony PCR using P9 and P10 primers. Activity was confirmed using the cell lysate. Cells from a positive colony were stored as H4362.

gaaD integration

The *gaaD* gene was already available in a multicopy plasmid from previous work [not published]. *gaaD* under *TPI1* promoter was amplified from B5470 using P9 and P10 primers, which added a *BglII* restriction site to each end of the fragment. The PCR *TPI1*/*gaaD* fragment and the vector pRS405 (containing Leu2 yeast marker) were digested with *BglII*, purified from gel and ligated. The DNA was electroporated into *E. coli* then plated onto LB-amp medium; positive colonies were pre-selected by colony PCR and sequenced with the primers P11 and P12 (for the *gaaD* ORF). A positive colony was stored as B5555.

The *gaaD* integration cassette was obtained by the linearization of B5555 using *AflII*. The *gaaD* cassette was integrated into the *gaaC*-expressing strain H4362 using the Gietz protocol. Transformants were selected in SCD - LEU (synthetic complete medium, without leucine, supplemented with 20 g D-glucose) and confirmed by colony PCR using P11 and P12 primers. The activity of the *gaaD* gene was confirmed using the cell lysate. A positive colony was stored as H4410.

*gaaA** integration

The *gaaA** gene was custom-synthetized as a codon-optimized ORF (Genescript). Both plasmid B2159, which contains the *TPI1* promoter region, and the plasmid B5706, which is the Genescript plasmid containing *gaaA**, were digested with *EcoRI* and *BamHI*. The *gaaA** fragment was ligated to the *TPI1* promoter in the opened B2159 plasmid. Colony PCR was performed with P13 and P13 to preselect the transformants. Later, a correct plasmid, confirmed by sequencing the *gaaA** ORF, was stored as B5696.
The integration plasmid was made as follows: amplification of the *gaaA** gene between the *TPI1* promoter and the terminator region, digestion of both the *TPI1*/*gaaA** fragment and the vector pRS406 (B704) with *BglII*, and ligation of the amplification fragment and plasmid. Colony PCR was performed with P13 and P14 to preselect the transformants. Later, a correct integration plasmid, confirmed by sequencing the *gaaA** ORF, was stored as B5697.

The *gaaA** integration cassette was obtained by the linearization of B5697 using *BsmI*. The *gaaA** cassette was integrated into the *gaaC* and *gaaD* expressing strain H4410 using the Gietz

protocol. Transformants were selected in SCD - URA medium and confirmed by colony PCR using P13 and P14 primers. Activity of the galacturonate reductase, corresponding to the *gaaA** gene was confirmed using the cell lysate. A positive colony was stored as H4425.

lgd1 integration

The integration plasmid carrying the *lgd1* gene between the *PGK1* promoter region and the *KanMX* yeast marker (resistance to G418) between *loxP* sites, was available from previous work [12] and stored as B3033. The plasmid was digested with *SacI* and *KpnI* and the integration cassette was purified from gel. The *lgd1* cassette was integrated into the *gaaC, gaaD* and *gaaA** expressing strain H4425 using the Gietz protocol. Before plating, the yeast cells were incubated in 2 mL of SCD for 1 h. Transformants were selected in YPD + G418 and confirmed by assaying *lgd1* activity of the cell lysate. The KanMx marker was removed by transforming the colony with plasmid pSH66, expressing Cre recombinase. The Gietz protocol was used for the transformation. Before plating, the yeast cells were incubated in 2 mL of YPD + nourseothricin for 1 h. After 4 days, the resulting colonies were re-plated in YPD + nourseothricin. Biomass from the plate was inoculated into 50 mL of YP + 2% galactose and incubated overnight. The resulting culture was plated in YPD. Some isolated colonies were replated in G418 plates and SCD -URA/-LEU/-HIS medium. After 2 days, none of the colonies grew on G418, confirming that the KanMix marker had been removed. A colony expressing *gaaC*, *gaaD*, *gaaA** and *lgd1* was stored as H4531.

gat1-gfp integration

The plasmid Genescript carrying the *gat1* transporter gene with *gfp* attached as a reporter gene and the plasmid B2159, which contains the *TPI1* promoter region, were digested with *EcoRI* and *NheI*. The fragment containing *gat1-gfp* was ligated to the open B2159 vector within the *TPI1* promoter region. Colony PCR was performed with P15 and P16 to preselect the transformants. Later, a correct plasmid, confirmed by sequencing the *gat1-gfp* ORF, was stored as B6367.

For making the integration cassette, the *TPI1*/*gat1-gfp* fragment was amplified from the B6366 plasmid using P17 and P18 and digested with *XmaI*. The vector which contains HO integration

region and *KnMX* yeast marker, was digested with *Cfr9I* and ligated to the *TPI1*/*gat1-gfp* fragment. Colony PCR was performed with P15 and P16 to preselect the transformants. Later, a correct integration plasmid, confirmed by sequencing the *gat1-gfp* ORF, was stored as B6382.

The *gat1-gfp* integration cassette was obtained by the digestion of B6382 with *XhoI* and *XbaI*. Transformation was done using the Gietz protocol. Before plating, the yeast cells were incubated in 2 mL of YPD for 2 h. Cells were plated in YPD + G418 plates. To confirm the integration of the *gat1-gfp* gene, cells were incubated in SCD medium for 24 h and analysed using a fluorescence microscope. A colony showing green fluorescence (from the *gfp* reporter gene) was stored as H4535.

Media and culture conditions

For plasmid multiplication, bacterial strains were cultivated in Luria Broth (LB) [13], supplemented with 100 μg mL^{-1} of ampicillin, at 37°C and 250 rpm. For yeast transformations, several media were used. SCD: synthetic complete medium, supplemented with 20 g D-glucose [14]. SCD - URA: uracil deficient synthetic complete medium, supplemented with 20 g D-glucose. SCD - HIS: histidine deficient synthetic complete medium, supplemented with 20 g D-glucose. SCD - LEU: leucine deficient synthetic complete medium, supplemented with 20 g D-glucose. SCD - URA/-HIS/-LEU: uracil, histidine and leucine deficient synthetic complete medium, supplemented with 20 g D-glucose. YPD: 10 g L^{-1} yeast extract, 20 g L^{-1} peptone, 20 g L^{-1} D-glucose [15]. YPD + G418: YPD supplemented with 200 μg mL^{-1} geneticin. YPD + nourseotricin: YPD supplemented with 100 μg mL^{-1} nourseotricin. For assaying D-galacturonic acid consumption by transformed yeast, the following medium was used: YP (10 g L^{-1} yeast extract, 20 g L^{-1} peptone) pH 4,5, supplemented with 12 g L^{-1} D-galacturonic acid; YP pH 4,5, supplemented with 12 g L^{-1} D-galacturonic acid and with 20 g L^{-1} fructose; YP pH 4,5, supplemented with 12 g L^{-1} D-galacturonic acid and with 50 g L^{-1} fructose.

Enzymatic assays

Cell extracts, obtained as follows, were used in all the enzymatic assays. Yeast cells were grown in YPD medium and collected by centrifugation, washed with ultrapure water and re-suspended in phosphate buffer 50 mM, pH 7 with addition of protease inhibitor Complete EDTA Free

(Roche, Switzerland). Cells were disrupted with 0.4 mm diameter glass beads using a bead beater FastPrep (MP Biomedicals, USA) and solid residues were removed by centrifugation. Protein content was assayed by the Bradford method [16].

GAAA and GAAD activities were assayed in the forward direction by following the decrease in absorbance at 340 nm caused by the oxidation of NADPH or NADH, respectively. The reaction medium for GAAA contained 10 mM D-galacturonic acid (Sigma-Aldrich, Germany) and 0.2 mM NADPH (Sigma-Aldrich, Germany). The reaction medium for GAAD contained 5 mM L-glyceraldehyde (Sigma-Aldrich, Germany) and 0.2 mM NADH (Sigma-Aldrich, Germany). The reaction was followed for 5 min.

LGD1 and GAAC activities were indirectly assayed by measuring the absorbance at 549 nm, corresponding to a chromogenic compound that forms when tiobarbituric acid combines with 2-keto-3-deoxy-galacturonate [17]. The reaction medium for LGD1 contained 10 mM L-galactonic acid (Sigma-Aldrich, Germany). The reaction proceeded for 2 h. The reaction medium for GAAC contained 10 mM L-glyceraldehyde (Sigma-Aldrich, Germany) and 10 mM pyruvate (Sigma-Aldrich, Germany). The reaction proceeded for 20 min. LDG1 was assayed in the forward direction. GAAC was assayed in the reverse direction as its substrate, 2-keto-3-deoxy-galacturonate, is not commercially available.

Chemical analysis

Samples of liquid fermentation broth were taken at 24 h intervals, with cells being removed by centrifugation and filtration through a 0.45 μm PVDF membrane. The concentrations of D-galacturonic acid, ethanol, fructose and glycerol were determined by HPLC using a 1260 Infinity Bio-Inert Quaternary LC System (Agilent, USA) fitted with a Cation-H pre-column linked to an ion exchange column Hi Plex H (Agilent, Santa Clara, California, USA) maintained at 65°C. The eluent was 5 mM H_2SO_4 at a flow rate of 0.55 mL min^{-1}. Peaks were detected using a differential refractometer at 40°C.

Results

Pathway assembly and gene expression

The reductive pathway for the catabolism of D-galacturonic acid was expressed in *Saccharomyces cerevisiae* CNPK11314C strain, with the genes being selected from three different filamentous fungi, *Aspergillus niger*, *Trichoderma reseei* and *Neurospora crassa*.

The first strategy that we tried was to integrate the four genes from *A. niger* (*gaaA*, *gaaB*, *gaaC* and *gaaD*), with each one being expressed under strong and constitutive yeast promoters (*PGK1* or *TPI1*). The last two genes, *gaaC* and *gaaD*, were integrated first and the corresponding enzyme activities were detected in the cell lysate. The next gene introduced into *S. cerevisiae*, *gaaA*, did not lead to observable activity of the galacturononate reductase. Thus, a codon-optimized ORF was custom-synthetized and re-integrated into the *S. cerevisiae* CNPK strain expressing *gaaC* and *gaaD*; this time, the activity of the corresponding enzyme was appreciable. The activity of the enzyme corresponding to *gaaB* also was not detectable after integration of *gaaB*, even when a codon-optimized ORF was used. Therefore, *lgd1*, from *T. reseei* was used instead, with success. The resulting strain, expressing *gaaC*, *gaaD*, *gaaA** and *lgd1* (H4531), was then re-tested for the activity of all four enzymes. The activities of these enzymes in the cell lysate obtained from H4531, expressed in terms of ncat/mg, are listed in Table 14.

Table 14. Enzyme activities assayed from S. cerevisiae H4531 cell lysate

Enzyme	Gene	Spec. Act. (nkat/mg)
EC 1.1.1.365	*gaaA**	0,246
EC 4.2.1.146	*lgd1*	0,018
EC 4.1.2.B7	*gaaC*	0,274
EC 1.1.1.372	*gaaD*	1,139

During the course of the study, a D-galacturonic acid transporter (coded by *gat1*) was described in *N. crassa* [18] and, hence, included in the integration strategy. This was done because, although a previous study reported that native *S. cerevisiae* is able to transport D-galacturonic acid when grown at acidic pH values, we felt the membrane transporter *GAT1* would improve the rate of consumption of D-galacturonic acid. For this reason, g*at1*, co-expressing *gfp* as a

reporter gene, was integrated into H4531, resulting in the strain H4535. *gat1* activity was not determined, but the transporter location was confirmed by fluorescence microscopy, with the green fluorescence of GFP being observed in the cellular membrane.

D-galacturonic acid consumption

Both recombinant strains, H4531 and H4535, were cultivated for 5 days under aerobic conditions in YP medium, pH 4.5, supplemented with 12 g L^{-1} of D-galacturonic acid. The original CNPK strain was used as a control. Even with evolutionary screening, the recombinant strains, as well the wild type, grew poorly and did not consume D-galacturonic acid. A second fermentation was therefore done, with the supplementation of the YP + galacturonic acid medium with 20 g L^{-1} of fructose (Fig 30).

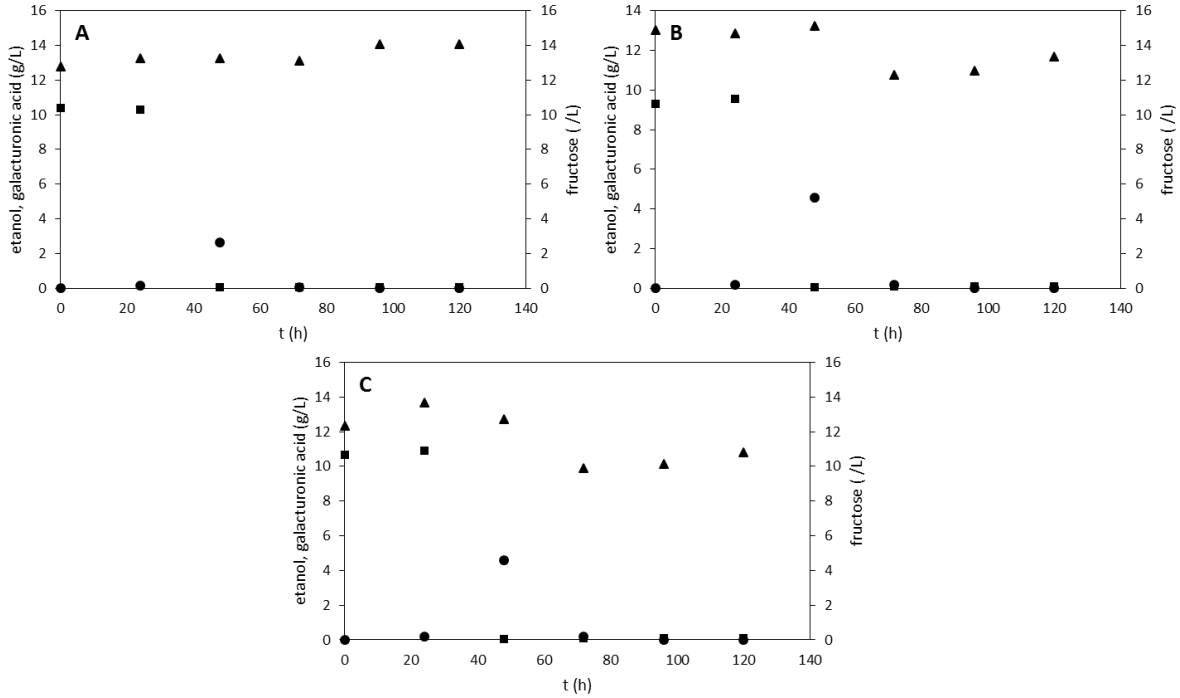

Figure 30. Cultivation of the yeast strains H2811 (wild type) (A), and the recombinant strains expressing the reductive pathway of the galacturonic acid catabolism H4531 (B) and H4535 (C) in YP pH 4.5 + 12 g L-1 D-galacturonic acid + 20 g L-1 fructose
Key: (■) fructose; (●) ethanol; (▲) D-galacturonic acid.

This time, D-galacturonic acid was consumed, with the two recombinant strains showing quite similar profiles. Consumption of D-galacturonic acid was negligible in the control fermentation.

A third fermentation was done with the fructose concentration being increased to 50 g L^{-1} (Fig 31). This time, the control strain did consume some D-galacturonic acid, but the consumption by the recombinant strains was noticeably higher. Since higher initial fructose concentrations were associated with higher consumption of D-galacturonic acid, fructose may be required as a co-substrate for D-galacturonic acid consumption. However, this is not the only possibility: it is possible that ethanol was used as a co-substrate for D-galacturonic acid consumption. It should be noted that ethanol accumulated in the media between 24 and 48 h, even though the conditions were aerobic; this is due to respirofermentative metabolism caused by the high fructose content. The ethanol was then consumed, and the decrease in the D-galacturonic concentration coincided with this period of ethanol consumption.

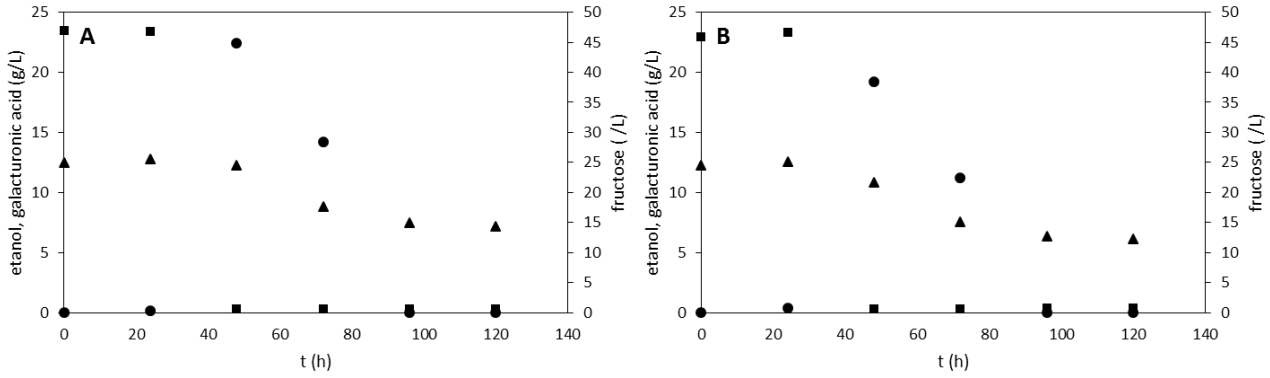

Figure 31. Cultivation of the recombinant yeast strains expressing the reductive pathway of the galacturonic acid catabolism H4531 (A) and H4535 (B) in YP pH 4.5 + 12 g L-1 D-galacturonic acid + 50 g L-1 fructose
Key: (■) fructose; (●) ethanol; (▲) D-galacturonic acid.

Interestingly, in the low pH media that were used, the strain H4535, which has the *gat1* gene integrated in addition to the four genes of the catabolic pathway, did not show any difference with respect to D-galacturonic acid consumption when compared with its counterpart H4531, in which only the four genes of the catabolic pathway are integrated.

Discussion

This work represents the first time that D-galacturonic acid consumption by *Saccharomyces cerevisiae* has been demonstrated, with all the enzymes of the D-galacturonic acid catabolic pathway being active in the cell lysate. However, this is not the first time that heterologous

expression of a pathway for D-galacturonic acid catabolism has been studied in this yeast. The two pioneering works in this area are discussed below.

Huijes et al. [19] integrated the five genes of the bacterial isomerase pathway (*uxaC*, *uxaB*, *uxaA*, *kdgK* and *kdgA*), which converts D-galacturonic acid to pyruvate and glyceraldehyde-3-phosphate. This pathway was first described between 1950 and 1960 [20-22] and consists of five enzymes (Fig 32A). The first enzyme, coded by *uxaC*, is uronate isomerase (EC 5.3.1.12), which converts D-galacturonic acid to D-tagaturonate. The second, D-tagarunate reductase (EC 1.1.1.58), coded by *uxaB*, reduces D-tagaturonate to D-altronate, using an NADH. Then, altronate dehydratase (EC 4.2.1.7), coded by *uxaA*, converts D-altronate to 2-keto-3-deoxy-D-gluconate. After that, 2-keto-3-deoxy-D-gluconate kinase (EC 2.7.1.45), coded by *kdgK*, uses an ATP to add a phosphate, forming 2-keto-3-deoxy-phosphogluconate. Finally, 2-keto-3-deoxy-phosphogluconate aldolase (EC 4.1.2.14), coded by *kdgA*, cuts the molecule to produce pyruvate and D-glyceraldehyde-3-phosphate.

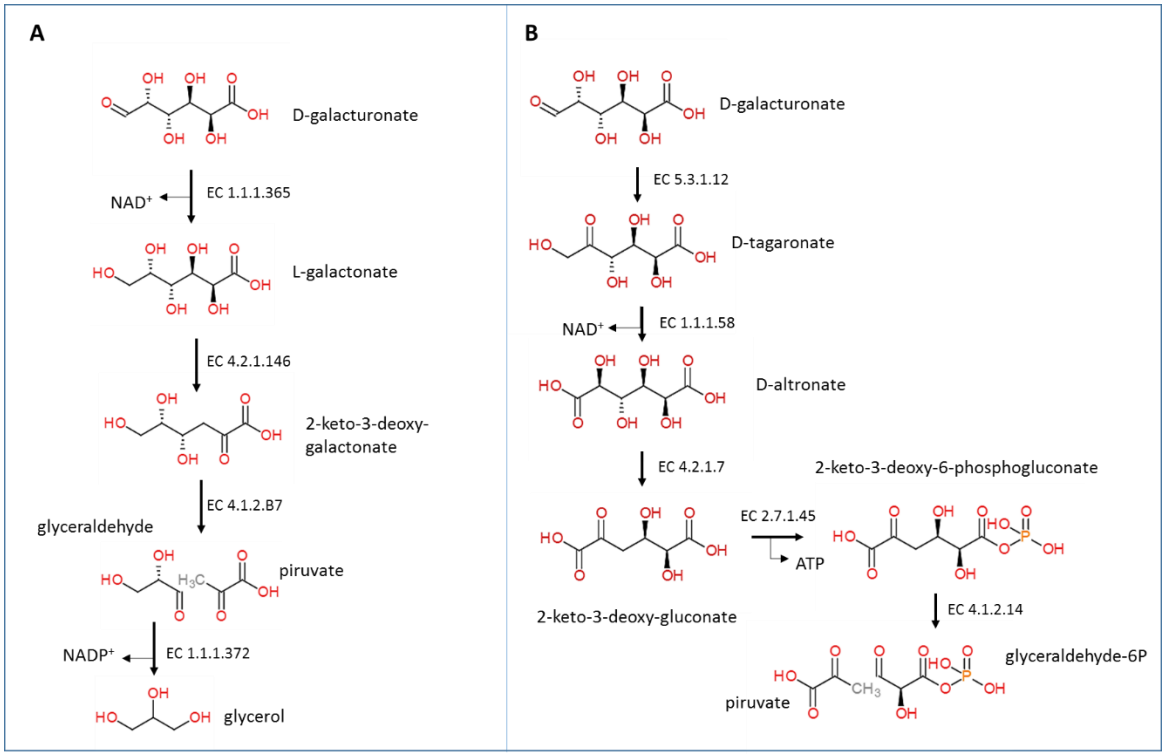

Figure 32. (A) Pathway of the bacterial isomerase D-galacturonic acid catabolism. (B) Pathway of the fungal reductase D-galacturonic acid catabolism

Although Huijes et al. [19] demonstrated by qPCR that all genes were transcribed, only two enzymes of the pathway showed detectable activity after integration in *S. cerevisiae*. Even with

adaptive laboratory evolution for 8 weeks in several media containing D-galacturonic acid, no consumption of D-galacturonic acid occurred. Huijes et al. [19] suggested that the failure of their strategy was due to the large genomic distance between bacteria and yeast; this distance makes it difficult to obtain successful expression of bacterial enzymes in yeast.

Souffriau [23] also tried, and failed, to express a pathway for D-galacturonic acid catabolism in *S. cerevisiae*, by cloning the four genes of the reductive fungal pathway. This reductive pathway was recently discovered by Hilditch et al. [24] and confirmed by Martens-Uznova et al. [25]. It consists of four enzymes, with two reduction steps (Fig 32B). The first enzyme of the pathway, D-galacturonate reductase (EC 1.1.1.365), coded by *gaaA* in *A. niger*, converts D-galacturonate into L-galactonate, using NADH as the electron donor. L-galactonate dehydratase (EC 4.2.1.146), coded by *gaaB* in *A. niger* and by *lgd1* in *T. reseei*, converts L-galactonate into 2-keto-3-deoxi-galactonate. The third enzyme, 2-keto-3-deoxi-galactonate aldolase (EC 4.1.2.B7), coded by *gaaC* in *A. niger*, cuts 2-keto-3-deoxi-galactonate between carbons 3 and 4, producing pyruvate and L-glyceraldehyde. The pyruvate can enter the citric acid cycle, while the L-glyceraldehyde is converted to glycerol by glyceraldehyde reductase (EC 1.1.1.372), coded by *gaaD* in *A. niger*, using NADPH as the electron donor.

Souffriau [23] cloned the genes of this reductive pathway from *Trichoderma reseei* (*gar1*, *lgd1*, *lga1* and *gld1*) into *S. cerevisiae.* Instead of integrating the four genes into the genome, he cloned the genes into two bidirectional vectors, under *gap-DH-ADH1* bidirectional promoters. Differently from Huijes et al. [19], he detected activity for all the genes in the cell lysate. Despite this, the recombinant yeast strain did not grow in D-galacturonic acid. Further, adaptive laboratory evolution, in a medium containing both glycerol and D-galacturonic acid, did not lead to mutants that were able to use D-galacturonic acid.

Similarly to Souffriau [23], we chose the reductive pathway for integration into *S. cerevisiae*, since previous work showed that the individual enzymes could be expressed in this yeast [12,24]. However, our approach differs from that of Souffriau [23] in four ways: First, we integrated the genes into the yeast genome, instead of using plasmidial vectors; second, we used genes from both *T. reseei* and *A. niger*; third, we also integrated a D-galacturonic acid transporter; and, fourth, we used fructose, instead of glycerol, as a co-substrate for growth of the recombinant yeast. Interestingly, both Souffriau [23] and we detected activity of all enzymes

in the cell lysate, but our use of fructose as a co-substrate, rather than the glycerol used by Souffriau [23], may be the main reason why we were able to demonstrate D-galacturonic consumption.

Conclusion

We constructed a strain of *S. cerevisiae* in which the four genes of the fungal reductive pathway for D-galacturonic acid catabolism were integrated into the genome and we observed consumption of D-galacturonic by this strain in a medium containing fructose. This represents the first step in the construction of a strain of *S. cerevisiae* that would be able to produce ethanol from D-galacturonic acid. Such a strain would find application in a citrus waste biorefinery, in which pectin-rich wastes would be hydrolyzed and then fermented to produce ethanol.

Acknowledgments

This research was supported by a “Universal” grant from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), a Brazilian government agency for the advancement of science and technology and by International Cooperation Project Grant, CNPq (Brazil) & AKA (Academy of Finland) (Finland), www.aka.fi/eng. Research scholarships were granted to David Mitchell and Nadia Krieger by CNPq, to Alessandra Biz by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) a Brazilian government agency for the development of personnel in higher education and to Alessandra Biz and Maura Sugai-Guerios by CNPq (SWE).

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Supplementary figures: Integration vectors

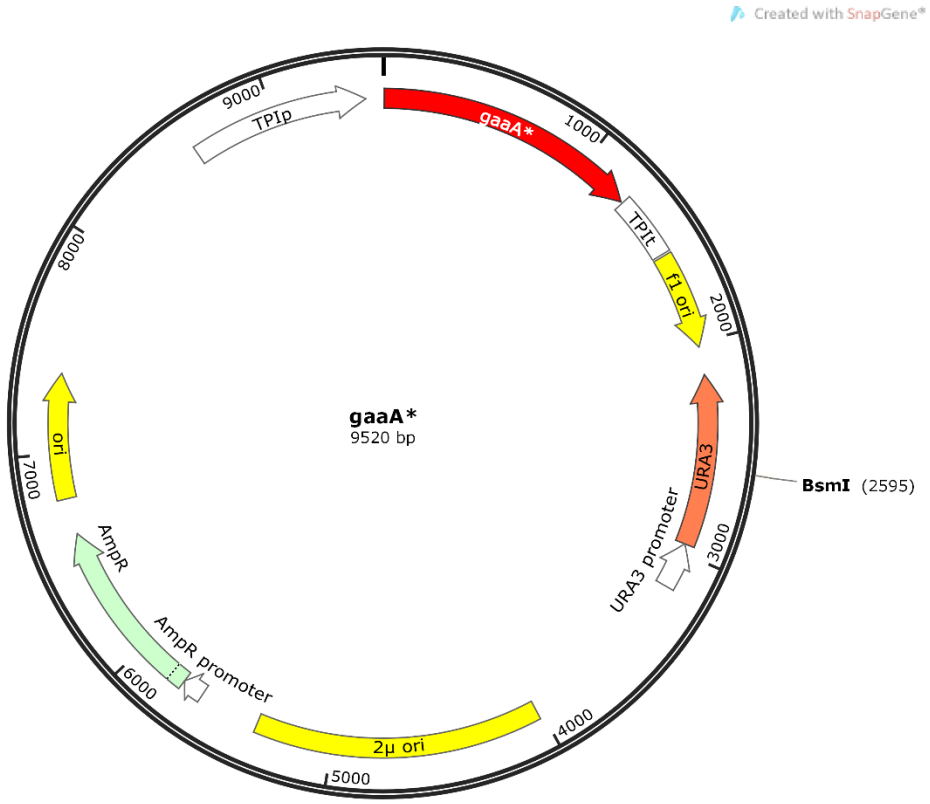

Figure 33. *gaaA** integration vector

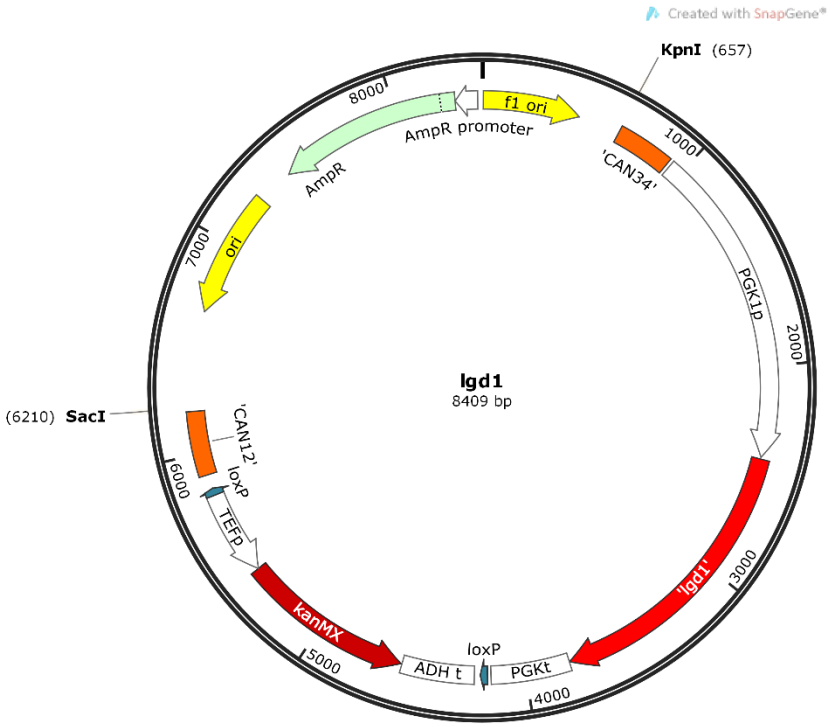

Figure 34. *lgd1* integration vector

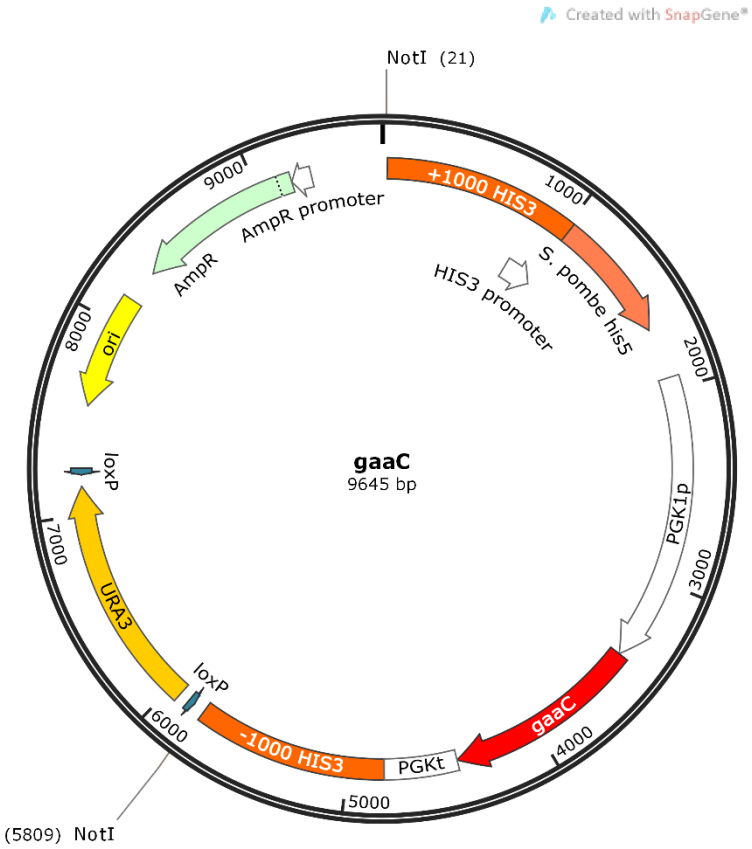

Figure 35. *gaaC* integration vector

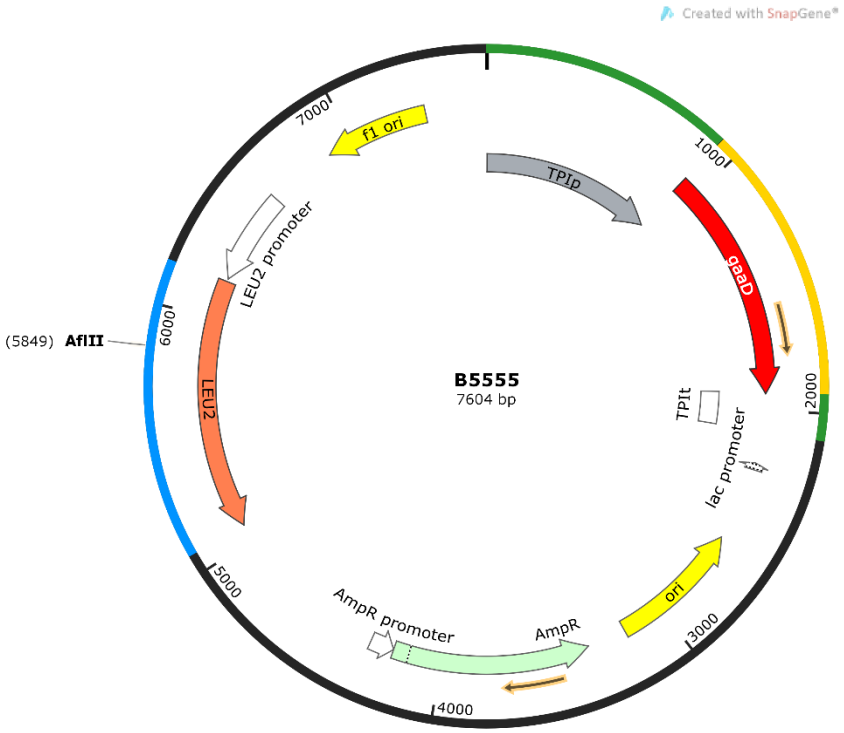

Figure 36. *gaaD* integration vector

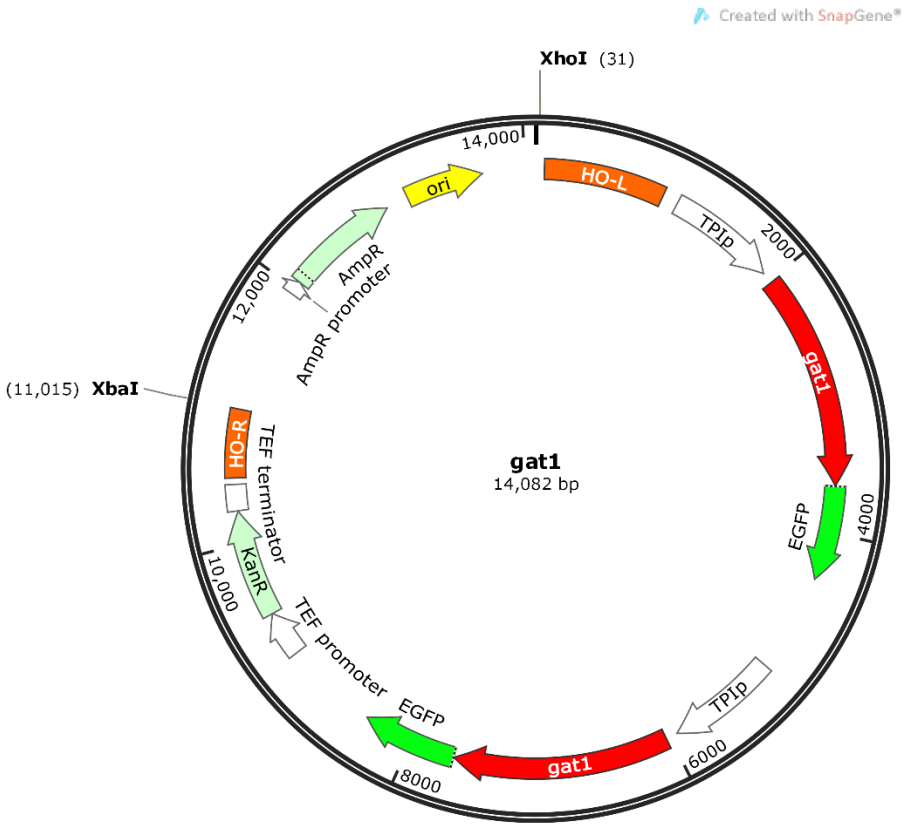

Figure 37. *gat1-gfp* integration vector

Considerações finais

Esta tese de doutorado visou contribuir para o desenvolvimento de soluções para os principais desafios de biorrefinarias de polpa cítrica. Contribuições relacionadas a três processos diferentes de uma biorrefinaria foram obtidas na tese:

(1) Hidrólise enzimática de pectina

No primeiro artigo (capítulo 2), ficou demonstrado, pela primeira vez, que existia uma desaceleração significativa na liberação de açúcares redutores nos primeiros minutos de hidrólise de pectina por complexos pectinolíticos (artigo 1). Esta descoberta tem implicações importantes para o desenvolvimento de processos de hidrólise enzimática de pectina: para otimizar estes processos, será necessário entender as causas desta desaceleração. De fato, esse trabalho inspirou um outro estudo que buscou desvendar as causas para essa desaceleração inicial (Farias, 2014), onde foi levantada a hipótese de que a desaceleração se deve à quebra rápida dos polímeros, com o ataque subsequente nos oligômeros liberados sendo mais lento. Além disso, o primeiro artigo apontou a falta de padronização dos ensaios para a determinação da atividade pectinolítica com relação ao tipo de substrato (pectina ou ácido poligalacturônico), à concentração de substrato utilizada, ao tempo e à temperatura do processo. Essa falta de padronização não tinha sido reconhecida explicitamente na literatura. É importante, porque torna difícil a comparação entre os diferentes processos de produção de pectinases descritos na literatura, e consequentemente, torna difícil a identificação de qual processo de produção de pectinases, entre aqueles relatados na literatura, seria mais adequado para a utilização em biorrefinarias.

(2) Produção de pectinases por fermentação em estado sólido

No segundo artigo, ficou demonstrado, em escala piloto com 15 Kg de substrato seco, que é possível produzir pectinases por fermentação em estado sólido a um baixo custo, utilizando resíduos agroindustriais, incluindo a própria polpa cítrica, como substratos. O substrato tinha quase 50% de sua massa composta por bagaço-

de-cana, que conferiu uma alta porosidade e estrutura estável no leito. Com isto, foi possível evitar totalmente o problema de superaquecimento do leito, um problema comumente relatado para processos de fermentação em estado sólido realizados em escalas maiores do que 1 Kg, incluindo a produção de pectinases utilizando um substrato baseado em farelo de trigo (Pitol et al. 2016). Além disso, foi realizado um teste de “prova-de-conceito”, com o uso direto do sólido fermentado produzido em escala piloto como catalisador da hidrólise de pectina cítrica, ou seja, foi evitada a necessidade de processos de separação/purificação das enzimas.

(3) Construção de uma cepa de *Saccharomyces cerevisiae* capaz de consumir ácido D-galacturônico

O terceiro artigo relata a construção, pela primeira vez, de uma linhagem recombinante de *Saccharomyces cerevisiae* capaz de consumir ácido D-galacturônico. Isto foi conseguido pela integração de uma via heteróloga de catabolismo de ácido D-galacturônico. A importância desta contribuição reside no potencial de usar o hidrolisado de polpa cítrica para a produção de etanol numa biorrefinaria: o ácido D-galacturônico representa cerca de 18% dos açúcares neste hidrolisado, mas o ácido D-galacturônico não é fermentescível por linhagens selvagens de *S. cerevisiae*, o organismo mais apropriado para a produção de etanol. A linhagem construída neste trabalho ainda não foi otimizada para a produção de etanol, mas representa um passo importante na direção da utilização de resíduos ricos em pectina para a produção deste biocombustível.

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